UNIVERSITY OF THESSALY

SCHOOL OF HEALTH SCIENCES

FACULTY OF VETERINARY MEDICINE

DEPARTMENT OF MICROBIOLOGY AND PARASITOLOGY

# THE ROLE OF EUROPEAN BROWN HARE (*LEPUS EUROPAEUS*) AS A SOURCE OF EMERGING AND RE-EMERGING PATHOGENS

A thesis submitted for the degree of Doctor of Philosophy

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"...Ithaka gave you the marvelous journey.

Without her you would not have set out.

She has nothing left to give you now.

And if you find her poor, Ithaka won't have fooled you.

Wise as you will have become, so full of experience,

you will have understood by then what these Ithakas mean…"

(C.P. Cavafy, Collected Poems. Translated by Edmund Keeley and Philip Sherrard.
Edited by George Savidis. Revised Edition. Princeton University Press, 1992)

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# INTRODUCTION

## THE EUROPEAN BROWN HARE *(LEPUS EUROPAEUS)*

### Taxonomy and Genetics

*Lepus europaeus* belongs to the order *Lagomorpha*, Family *Leporidae*, Genus *Lepus* with 31 other hare and jackrabbit species. The taxonomy of the genus *Lepus* is fundamentally difficult to deal with as it is young and rapidly expanding while its open grassland habitat becomes widespread through the activity of pastoralists and agriculturalists. Coupled with that is their great mobility as hares can travel up to 15km in a night and populations may shift hundreds of kilometers if driven by snow or drought (Chapman et al., 1990).

The knowledge of *L. europaeus* genetic diversity is an important tool for understanding the population structure and dynamics of this species under different environmental conditions. Although it is quite widespread in Europe, studies related to its population structure based on molecular markers are limited.

A population genetic study based on allozymes, showed that genetic diversity was highest in Anatolian hares, intermediate in brown hares from the southern and southeastern Balkans and lowest in central European populations. The authors suggested that the rich genetic diversity in Anatolian hares might have been a consequence of Anatolias biogeographic position due to multiple gene flow from neighboring regions and the long-term presence of hares during the last ice age, when large parts of more northern latitudes did not provide suitable habitats (Sert et al., 2005). In another study, allozyme variability of *L. europaeus* from Greece was compared to the existing data of Bulgarian populations. This study showed the presence of three alleles at low frequencies in Greek hares, which were not found in Bulgarian or any other hare population. On the other hand, some alleles from the Bulgarian and other European populations were absent in the Greek hares. Moreover, the data analysis revealed increased gene pool diversity in Greek hares and slight gene flow between the two neighboring regions. These findings conformed to the hypothesis of the existence of a Late Pleistocene refugial population in the southern Balkans presenting a few specific nuclear gene pool characteristics, but with little effect on the overall genetic differentiation between Greek and Bulgarian hares (Suchentrunk et al., 2003).

In a recent population genetic study, *L. europaeus* samples from Greece, Bulgaria, Italy, Croatia, Serbia, Poland, Switzerland, Austria, France, Germany, the Netherlands, Spain, the United Kingdom, Turkey and Israel were analysed based on mitochondrial DNA (mtDNA) indices. The haplotypes detected were partitioned into five phylogeographically well-defined major haplogroups. In Bulgaria and north-eastern Greece numerous haplotypes of all five haplogroups were present. A large overlap zone was identified, and this part of the south-eastern Balkans was defined as the region with the highest mtDNA nucleotide diversity. This study suggested that gene flow occurred from Anatolia to Europe across the late Pleistocene Bosporus land-bridge (Stamatis et al., 2009).

Previously, Kasapidis et al., 2005 analysed the phylogeographic structure of *L. europaeus* from continental and insular Greece, Bulgaria, Cyprus and northern Israel, together with other published sequences from Italy and central Europe based on mitochondrial DNA (mtDNA) indices. This study revealed the existence of two distinct phylogenetic clades a “west” and an “east” type. Hares from mainland Greece and the Greek islands (Lefkada, Naxos, Kythira and Crete) and Bulgaria, Italy, and central Europe (i.e., Germany, Austria, Hungary, Serbia, Romania), clustered within “west type”, while hares from Bulgaria, the Greek prefectures of eastern Macedonia and Thrace, the eastern Aegean islands of Lesvos, Chios, Samos and Rhodes, as well as Cyprus and northern Israel clustered within “east type”. These two types formed an overlap zone in the area of Thrace and Bulgaria.

Hare populations from central Greece were studied and specific mtDNA profiles were identified which clearly differentiated reared from wild individuals. The mtDNA profiles showed extensive haplotype diversity within and among wild populations and proved highly indicative for reared hares from past releases which were caught within wild populations. This finding suggested the introgression of allochthonous gene pools into the native populations (Mamuris et al., 2001). Although prior to 2003, no captive-bred central European haplotypes had been detected in wild Greek hares (Mamuris et al., 2001; Stamatis et al., 2009) analyses based on samples collected after 2003 (Spyrou et al., 2013; Stamatis et al., 2007) detected 29 hares with haplotypes matching those of previously analysed captive-bred individuals thus providing evidence that some of

these hares had survived long enough and/or they had at least one reproductive cycle and, thus, transmit their genomes.

## Distribution, Population, Habitat

*L. europaeus* is probably the most important game animal in Europe throughout its historical distribution, including those areas where it has been introduced, being widespread and abundant across its geographic range. The current Eurasian distribution of *L. europaeus* extends from northern Spain, to introduced populations in the United Kingdom and southern Scandinavia, south to northern portions of the Middle East, and has naturally expanded east to Siberia (IUCN, 2008). This species has been introduced as a game species extensively to countries including Argentina, Australia, Barbados, Brazil, Canada, Chile, Falkland Islands, New Zealand, the United Kingdom and the United States (Chapman et al., 1990).

Decline in the populations of *L. europaeus* has been experienced in many areas across its geographic range in Europe, beginning in the 1960s, in association with the intensification of agricultural practices (Smith et al., 2005). There is considerable concern over recent declines in Europe, but the populations are not really threatened. Hare population densities range from 0.1/ha to 3.4/ha (IUCN, 2008). The decline of this species population has garnered its protection under the Bern Convention as an Appendix III listing (Vaughan et al., 2003). However, in Norway, Germany, Austria and Switzerland, population declines have resulted in country-specific Red Listing as "near threatened" or "threatened" (IUCN, 2008).

## Ecology, Behavior, Reproduction and Diet

*L. europaeus* is a highly adaptable species that can persist in various habitat types. High arctic tundra, steppe, agricultural pasture, tropical savanna and desert are all types of habitats occupied by hares. A positive association has been found with hare abundance and habitat density and diversity. However, the unifying aspect of habitats preferred by hares is open country. Some form of cover like shrubs, bushes or even rocks is needed for their protection from birds of prey as unlike rabbits, which run to cover for protection, hares tend to use cover for shelter by day and run into the open to avoid predators when attacked (Smith et al., 2005).

This species keeps the so-called home range; individual home ranges vary from 10 to 300 ha and often overlap on favored feeding areas. Hares usually travel up to 1.8 km in search of suitable graze. However, it has been reported that they may travel up to 15 km in one night whilst feeding (Chapman et al., 1990). They can be found at elevations ranging from sea level up to 2.300 m (IUCN, 2008).

Hares appear to be essentially silent, solitary animals. Though, complex behavioral interactions may explain the regulation of population density at levels normally far below the carrying capacity of the environment. There is no indication of territorial behavior, although a dominance hierarchy between individuals has been demonstrated for food (Lindlof, 1978; Monaghan and Metcalfe, 1985). They are mainly open country grazers, relying on speed to elude their many enemies. Although largely nocturnal, they may start feeding in mid-afternoon in higher latitudes and in the breeding season. Feeding takes about a third of a hare's time, and before entering its "form" for the day it backtracks to confuse predators (Chapman et al., 1990).

Reproductive behavior reaches a peak in spring (Mad March hares) with up to a dozen males congregating round a female in oestrus, boxing each other and chasing off rivals. Gestation is 41-42 days and reproduction occur year-round. Females give birth to their young in open areas, or in a shallow depression in the ground. *L. europaeus* averages three litters/yr but can vary from one to four litters/yr. Leverets remain hidden within dense vegetation, and the female visits them in order to nurse them. Nursing takes place once or twice a day, in the late evening, and lasts only a few minutes (Broekhuizen and Maaskamp, 1980). Approximately three days after birth, leverets disperse to separate hiding locations. Leveret litter-mates will regroup for a brief suckling bout at a location at precisely defined intervals. Females reach maturity around seven to eight months and males at six months (Chapman et al., 1990). Average life expectancy for hares is 1.04 years, with a maximum age span in the wild of 12.5 years recorded in Poland (Chapman et al., 1990).

Hares are entirely vegetarian. Many types of grasses, shrubs and forbs can be eaten, and most hares are adaptable. The food eaten varies with the habitat; in Europe hares feed on agricultural land eating 90% of soft greens, 5.5% woody plants, 2.2% root crops, 1.7% grain crops and 0.5% forest plants. Lagomorphs are well adapted for obtaining the greatest possible value from their food. They produce two types of faecal material:

moist pellets and dry pellets. The moist pellets are expelled and then eaten; this is done with little or no chewing, and as a result the majority of the food passes through the digestive tract twice (coprophagy) (Chapman et al., 1990; IUCN, 2008).

### Threats

Lagomorphs make up the base of many predator-prey systems. Their intermediate size and great abundance put them in a position to support a community of small to medium-sized predators. Weasels, foxes, coyotes, cats, civets and many large birds of prey are all sustained by lagomorph populations (Chapman et al., 1990). In Europe, it has been shown that hare is a common prey for large birds of prey, canids and felids which comprise its natural predators (Alves et al., 2008). Recently, a study conducted in Greece showed that *L.europaeus* comprises one of the most important trophic groups in the diet of red foxes as it was consumed significantly more within hunting areas (Bakaloudis et al., 2015) while *Martes foina* rarely exhibits rapacity in the European brown hare (Bakaloudis et al., 2012).

## EUROPEAN BROWN HARE (*LEPUS EUROPAEUS*) AS A SOURCE OF SELECTED EMERGING AND RE-EMERGING PATHOGENS; A REVIEW

### Introduction

Infectious pathogens of wildlife may affect human health and livestock production while wildlife have long been recognized as potential source for emerging infectious diseases strongly associated with outbreaks that have serious consequences for animal and human health (Chomel et al., 2007; Thompson et al., 2009). The World Organization for Animal Health (OIE) defines an emerging disease as “a new infection or infestation resulting from the evolution or change of an existing pathogenic agent, a known infection or infestation spreading to a new geographic area or population, or a previously unrecognized pathogenic agent or disease diagnosed for the first time and which has a significant impact on animal or public health.” A known or endemic disease is considered to be re-emerging if it shifts its geographical setting, expands its host range, or significantly increases its prevalence.

The European brown hare is one of the most important game animal species in Europe. A decline of its population throughout its range has been noted since the 1960s (Posautz et al., 2015) whereas its causes are not fully understood. External factors such as climate, feed availability, diseases and predators, internal factors such as reproductive rate and ability for adaptation and anthropogenic factors may affect the population density of this species (Treml et al., 2007). To address population declines, release of captive-bred individuals into the wild has been carried out in several European countries. Though, criticisms of wildlife restocking operations concern the risk of pathogens introduction in new areas through translocations and the threat posed on the integrity of locally adapted genetic diversity (Spyrou et al., 2013). European brown hare is considered a susceptible bioindicator of environmental changes and its epidemiological importance stems for the fact that it maintains the so-called home range thus being prone to many infectious diseases of natural nidality (Treml et al., 2007). Besides, its short life span makes it an excellent indicator for recent pathogen transmission in the area and its intensive contact with vectors such as ticks, mosquitoes and sandflies has implicated hare in the epidemiology of vector borne diseases such as Leishmaniasis, and Crimean–Congo hemorrhagic fever virus (Dobler et al., 2006; Moreno et al., 2014). Apart from common diseases of hares with high impact on their mortality such as staphylococcosis, pasteurellosis, pseudotuberculosis and European Brown hare Syndrome, hare has been deemed as a source of important pathogens such as the agents causing tularaemia, listeriosis, toxoplasmosis, leptospirosis and borreliosis posing threat for human health especially when handling affected animals (Bartling et al., 2004; Frandölich et al., 2003; Treml et al., 2007, 2003). The present review provides an overview of the available information on selected viral, bacterial and protozoal emerging and re-emerging pathogens detected in European Brown hare.

## Viral pathogens

### European Brown Hare Syndrome Virus (EBHSV)

European brown hare syndrome virus (EBHSV), a small (30 to 35 nm) icosahedral, nonenveloped and hemagglutinating single-stranded–RNA virus, member of the genus

*Lagovirus* of the family *Caliciviridae* (International Committee on Taxonomy of Viruses, 2012) is the causative agent of European brown hare syndrome (EBHS). EBHS was diagnosed for the first time in 1980 during an epidemic of a fatal disease causing severe liver damage in hares in the island Gotland (Sweden) (Gavier-Widén and Mörner, 1991). The etiological agent was originally unknown but assumed to be either an infectious agent, most likely a virus, or a toxic chemical. In 1988, viral particles were identified as the causative agent by electron microscopy (Alves et al., 2008). Thereafter, the disease was reported in many European countries, including the United Kingdom, Poland, Switzerland, Sweden, Spain, Denmark, Italy, France, Belgium, Finland, Croatia and Greece (Alves et al., 2008; Billinis et al., 2005). Though, retrospective serological studies have demonstrated that the virus was present in Europe and other countries since as early as 1976 while hunters in Scandinavia knew of the disease in the early 1970s (Gavier-Widén and Mörner, 1991).

Even though oral or nasal transmission occurs, the oral-faecal route seems to be the most important one; the virus is shed in faeces of infected animals and it also being spread in the droppings of predators that have consumed infected hares resulting in the contamination of food and water (Alves et al., 2008; Gavier-Widén and Mörner, 1991). Besides, EBHSV is highly robust, resisting acid of pH3, and may remain infectious for 3–4 months in the field (Alves et al., 2008). Recently it was shown that red foxes fed on EBHSV infected hares, may have genetic prints of the virus in their gut contents suggesting their potential role in the indirect transmission of the virus (Chiari et al., 2016). Similarly, the potential role of wolf (*Canis lupus*) as a EBHSV passive carrier, favoring the introduction and spread of the virus among different hare populations has been suggested (Di Profio et al., 2018). Humans, insects, and birds can also act as vectors but no reservoir hosts have been yet identified (Alves et al., 2008).

The disease affects the species *L. europaeus* and *L. timidus* with a 35–80% mortality occurring 48–72 h after infection (Chasey et al., 1992). Morbidity and mortality rates are extremely high especially in adult individuals. The disease has not been observed in hares younger than 40–50 days while hares 2–3 months of age may contract infection but they do not usually develop disease (Paci et al., 2011). Mortality is highest in the autumn because of the higher population densities at the end of the breeding season, the unfavorable weather conditions and the increased susceptibility of the offsprings

following the reduction of the maternal protective antibodies (Drews et al., 2011; Scicluna et al., 1994). Hunting practice may also facilitate dissemination of the virus due to the increased presence of people and dogs in the natural hare habitat. Moreover, natal dispersal rates of hares are significantly higher in hunting areas compared to the non-hunting ones which might also contribute to the spread of the virus (Bray et al., 2007; Drews et al., 2011).

When a hare population contracts infection for the first time, an initial epidemic peak occurs during which, a decline in the hare population may take place. Thereafter, it tends to endemicity and in such areas the hare population appears to remain stable as most of the hares are immune and mortality rates are low (Alves et al., 2008; Scicluna et al., 1994). Newborn hares may have antibodies against EBHSV, indicating that there is colostral or transplacental passive transfer of immunity (Gavier-Widén and Mörner, 1991) while hares that survive infection develop long-lasting, protective immunity (Alves et al., 2008). The impact of infection on the hare population is highly dependent on the local population density; hare populations can be reduced dramatically following EBHS outbreaks when the hare density is low (<8 hares/km$^2$). It has been suggested that the mortality can be reduced and attain endemic stability by increasing the host density about 15 adults/km$^2$. In such cases, increased transmission and infection of hares at a young age (>3 months) subsequently results in the development of protective antibodies (Paci et al., 2011).

EBHS is characterized by rapid progression and death can be sudden, lacking any clinical signs. Behavior modifications could be observed frequently such as lack of fear, dullness, jumping into the air, circling, staggering, incoordination and convulsion before death (Duff et al., 1994). In farmed hares, it is also possible to observe anorexia, apathy alternated with excitement, cries and respiratory distress during agony. Chronic or subclinical course of the disease characterized by generalized jaundice at the mucosal and subcutaneous level has been also suggested in approximately 30–50% of hares during an outbreak of the disease. In such cases, the affected hares die after several days or finally recover (Alves et al., 2008). The principal findings during necropsy are serohemorrhagic liquid at the nostrils, haemorrhages on serosa and mucosa, oedema and congestion of tracheal mucosa with foamy haemorrhagic contents, liver enlargement, degeneration and decolouration with sharply demarcated and friable

lobes, enlargement of the spleen and generalized jaundice and congestion of the spleen and kidneys (Alves et al., 2008; Billinis et al., 2005; Duff et al., 1994; Frölich et al., 1996). Hepatic damage has a central pathogenetic role in EBHS (Marcato et al., 1991) while histological analysis of the liver is characteristic and diagnostic (Gavier-Widén, 1994).

Serological studies conducted in different European countries revealed a seroprevalence ranging from 0% in Switzerland to approximately 95% in Italy (Chiari et al., 2014; Eskens et al., 2000; Frandölich et al., 2003; Frölich et al., 2007, 2003a, 2001, 1996; Scicluna et al., 1994). At the same time, when using ELISA for the detection of EBHSV antigens, the prevalence reported ranged from 1.2% in Switzerland to 7.6% in Poland (Frölich et al., 1996). RT-PCR has been also used in previous studies for the detection of EBHSV nucleic acid; in Slovakia 18% of the examined hares were EBHSV infected while 73% of them were seropositive by ELISA (Frölich et al., 2007). In Finland 6% and in Greece 87.6% of the examined hares were considered as EBHS cases as they were RT-PCR positive and the histopathological findings were compatible with the disease (Billinis et al., 2005; Syrjälä et al., 2005).

Phylogenetic studies suggested that virus dispersion occurred from Sweden to west, east and southern Europe (Frölich et al., 2007). However, as already mentioned, EBHSV likely emerged in the early 1970s, as suggested by descriptions of hares with lesions consistent with the disease in 1976 in England while antibodies against the virus have also been found in archived sera, and the virus was detected by PCR in samples collected in Sweden before 1980 (Frölich et al., 2007; Le Gall-Reculé et al., 2006; Lopes et al., 2014a; Ros Bascuñana et al., 1997). Recently, phylogenetic analysis revealed that a single viral lineage was dominant at the time of EBHSV emergence which spread throughout Sweden and evolved in separated genetic subgroups that clustered in distinct geographical areas. This lineage persisted until 1989 when it was probably replaced by a new lineage that emerged in the mid-1980s. The new lineage included Swedish samples from the late 1980s and all the Swedish samples from the 1990s and 2000s, along with samples from other European countries (Italy, France and Germany). This finding was suggestive of an increased fitness which may have enabled the new lineage to diffuse over a wider geographic area along with the successful dispersal and persistence of this lineage throughout Europe (Lopes et al., 2014a).

EBHSV is closely related to the Rabbit Haemorrhagic Disease (RHD) virus with which it shares significant similarities in terms of their epidemiology, symptomatology and pathology. Though, viral genome sequencing revealed that EBHSV shares a similar genomic organization with RHDV, but that the two viruses are distinct. They also differ in their evolutionary patterns with a strongest geographical structure for EBHSV (Le Gall-Reculé et al., 2006; Lopes et al., 2014a).

### Rabbit haemorrhagic disease virus 2 (RHDV2)

Rabbit haemorrhagic disease virus (RHDV), a non-enveloped single-stranded positive-sense RNA virus belonging to the genus *Lagovirus* of the family *Caliciviridae*, is the causative agent of rabbit haemorrhagic disease (RHD), which only affects *Oryctolagus cuniculus* (domestic and wild rabbits). The disease is highly contagious and usually fatal, with mortality range of 70–100%. In 2010, a variant of RHDV was detected in France during RHD outbreaks in vaccinated rabbits (Le Gall-Reculé et al., 2011). Further studies on the variant indicated that it could not have evolved from RHDV but represented a new virus, leading to the proposal of the variant as a new lagovirus species, named RHDV2 (Le Gall-Reculé et al., 2013, 2011). This new virus has a unique antigenic profile from that of RHDV that it could be considered a distinct serotype. Moreover, longer disease duration, variable mortality rates (5–60%) and capacity to infect young rabbits (from 10 to 15 days of age) are among the specific and distinguishing characteristics of RHDV2 (Le Gall-Reculé et al., 2013; Velarde et al., 2016). The latter has been also reported to be rapidly spreading throughout Europe, and has caused significant losses in farmed and wild rabbits in France, Italy, Portugal, Spain, Germany, the United Kingdom and Australia (Abrantes et al., 2013; Baily et al., 2014; Dalton et al., 2012; Le Gall-Reculé et al., 2013, 2011; Puggioni et al., 2013; Velarde et al., 2016; Westcott et al., 2014)

RHDV and EBHSV, are considered strictly species-specific and restricted to *Oryctolagus* (rabbit) and *Lepus* (hare) genera, respectively (Lavazza et al., 1996). Just one report of RHDV in the Iberian hare (*Lepus granatensis*) exists (Lopes et al., 2014b) while field and experimental data recently have demonstrated that in Italy, the eastern cottontail (*Sylvilagus floridanus*) is susceptible to infection with EBHSV, occasionally resulting in EBHS-like disease (Lavazza et al., 2015). In contrary, RHDV2 causes fatal hepatitis in

European rabbits (Le Gall-Reculé et al., 2011), Cape hares (*Lepus capensis mediterraneus*) (Puggioni et al., 2013), and in Italian hares (*Lepus corsicanus*) (Camarda et al., 2014). Recently, RHDV2 has also been detected in European brown hares (*Lepus europaeus*) in Italy, Spain (Velarde et al., 2016) and Australia (Hall et al., 2017).

In the outbreak of RHDV2 in Sardinia, a significant number of Cape hares and rabbits were concurrently affected by the disease (Puggioni et al., 2013), indicating a quite high frequency of infection in Cape hares. In Iberian hare (Lopes et al., 2014b) and in both an Italian hare (Camarda et al., 2014) and European Brown hare (Velarde et al., 2016), the virus did not cause severe or easily detectable outbreaks, but rather only single, sporadic infections. Thus, it was suggested that the level of susceptibility differs among the hare species, with Italian hare probably being intermediate susceptible compared to Sardinian cape hare and European brown hare (Camarda et al., 2014).

The two RHDV2 strains detected in European brown hares caused macroscopic and microscopic lesions similar to EBHS, and they were genetically related to other RHDV2 strains in Europe (Velarde et al., 2016). European brown hares likely have been exposed to high levels of RHDV2 during outbreaks in rabbits in recent years. The fact that only sporadic infections of RHDV2 in European brown hares have been reported probably suggests that this species is not a primary host (Velarde et al., 2016) and acts as spillover host when infection pressure is high and barriers between rabbits and hares are limited (Velarde et al., 2016). The virus spillover to *L. europaeus* creates further concern as this species is more widely distributed thus making possible the wider and more rapid spread of RHDV2 and, at the same time, poses serious threat to vulnerable or endangered *Leporidae* species (Hall et al., 2017; Velarde et al., 2016). However, the virulence and associated case fatality rate of RHDV2 in *L. europaeus* compared with *O. cuniculus*, and their susceptibility to infection, has not yet been determined (Camarda et al., 2014; Hall et al., 2017; Velarde et al., 2016).

### Myxoma Virus (MYXV)

Myxomatosis is a major viral disease of wild and domestic European rabbits (*Oryctolagus cuniculus*) caused by the Myxoma virus (MYXV), a poxvirus (family *Poxviridae*; subfamily *Chordopoxvirinae*; genus *Leporipoxvirus*). MYXV was first isolated from a colony of laboratory rabbits in Uruguay in 1898 (MYXOMATOSIS; OIE Terrestrial

Manual 2014). The virus was introduced into Australia (1950), continental Europe (1952), Great Britain (1953) and Ireland (1954) as a biological control measure for wild European rabbits but now it has a worldwide distribution (MYXOMATOSIS; OIE Terrestrial Manual 2014) and it is endemic in wild European rabbit populations with the potential of spill over into farmed, laboratory and pet rabbits (Fenner and Fantini, 1999). *Sylvilagus brasiliensis* (South America) and *S. bachmani* (California, USA) are its natural hosts presenting few clinical signs (Osterhaus, 1994). MYXV is shed in ocular and nasal secretions or from skin lesions. However, the virus presence in semen and genital secretions has been also suggested. Transmission is made mainly by the bite of insects such as mosquitoes, fleas, midges and lice. In Great Britain the rabbit flea (*Spilopsyllus cuniculi*) is the most important vector of the virus while other blood-sucking insects may play a minor role as vectors in certain circumstances. Direct transmission may also occur when rabbits live in close proximity (i.e. in farmed animals) (OIE, 2014).

Two forms of the disease have been reported; the nodular (classical) form and the amyxomatous (respiratory) form. Nodular myxomatosis, mainly observed in wild and pet rabbits and in small-scale rabbitries, is characterized by florid skin lesions and severe immune dysfunction, accompanied by supervening bacterial infections of the respiratory tract. The mortality rate fluctuates between 20 and 100%, according to the grade of virulence of the viral strain (Grade I to V as determined in laboratory rabbits). Secondary bacterial infections (mainly *Pasteurella* sp. and *Bordetella* sp.) of the conjunctivae, upper respiratory tract, and lungs are common and may be the major cause of death in rabbits infected with subacute strains of MYXV. Amyxomatous myxomatosis is characterized mainly by respiratory clinical manifestations. However, fewer and smaller cutaneous lesions than the nodular type may also occur. The amyxomatous form is regarded as more significant for farmed rabbits as direct contact is needed for transmission, presumably via respiratory and conjunctival secretions. The virulence of amyxomatous viruses appears to depend on the presence of bacterial pathogens such as *Pasturella multocida* (Marlier et al., 2000; OIE, 2014).

Myxomatosis has been rarely reported in the European brown hare. It appears following a protracted course with multiple firm subcutaneous nodular masses at the head, back and limbs (Wibbelt and Frolich, 2005). In 1937, wild brown hares in Australia

were experimentally inoculated with virulent MYXV from rabbits without inducing overt disease (Barlow et al., 2014). Thereafter, a few cases were reported from France (Jacotot et al., 1954; Lucas et al., 1953; Magallon and Bazin, 1953) and Ireland (Collins, 1955; Whitty, 1955). The case reported in Ireland, did not present suggestive lesions of myxomatosis but when tissues from this hare were injected to rabbits, the disease was replicated thus suggesting that hares may be subclinically infected and probably act as carriers of the disease (Collins, 1955). Recently, a confirmed case was reported from Great Britain. The hare presented proliferative raised skin lesions at the base of both ears and eyes and the mid-lateral aspect of both front and hindlegs. The presence of virus particles in the affected skin lesions was confirmed by electron microscopy and the presence of the viral DNA was confirmed by PCR and sequencing. Secondary cutaneous *Staphylococcus aureus* infection resulting in toxaemia or septicaemia was suggested as the cause of death by the authors (Barlow et al., 2014).

### Hare fibroma virus

Fibromatosis is a disease typical of hares caused by a *Leporipoxvirus* in the family *Poxviridae*. The virus is antigenically related to the agent of rabbit (Shope) fibroma, squirrel fibroma and MYXV as shown by plaque-neutralization and cross-protection tests (Fenner, 1965; Leinati et al., 1961; Woodroofe and Fenner, 1965). Agar-gel diffusion microprecipitation techiques revealed that hare fibroma virus is antigenically more closely related to rabbit fibroma virus than MYXV (Fenner, 1965). Though, a considerable degree of cross-protection exists between hare fibroma virus and MYXV; rabbits immunized with MYXV are completely refractory to hare fibroma virus whereas rabbits immunized with hare fibroma virus develop myxomatosis, but survive infection (Woodroofe and Fenner, 1965).

The disease was first described in European Brown hare in France and Northern Italy in 1959 (Lafenetre et al., 1960; Leinati et al., 1959). Moreover, nodular skin disease of hares reported earlier in Germany which were designated as hare sarcoma, were probably fibromatosis (Suckow et al., 2012). Although fibromatosis is considered restricted in Europe, dermal tumors with histopathologic similarities to hare fibromatosis have been also reported on Cape hares (*Lepus capensis*) in Kenya (Karstad

et al., 1977). In 2001, an outbreak of fibromatosis occurred in farmed hares in Italy (Grilli et al., 2003).

The virus is probably mechanically transmitted by arthropods, but infection can also occur by direct contact (Grilli et al., 2003). The presence of skin microlesions or trauma is considered to be a predisposing factor. A seasonal occurrence of disease has been reported, with the peak incidence in late summer and autumn (Lafenetre et al., 1960; Leinati et al., 1959). Morbidity is usually high, but mortality is low. Most adult hares spontaneously recover within one to three months (Grilli et al., 2003).

The disease in hares is characterized by the development of single or multiple protruding skin nodules up to 2.5cm in size occurring mostly on the face, eyelids and around ears while fibromas on the legs were also reported in the outbreak of fibromatosis in Italy (Grilli et al., 2003; Suckow et al., 2012). The disease is endemic and of little significance in hares. Thus, no control measures have been developed. However, during re-stocking operations, hares should be free of infection to avoid dissemination of the virus (Suckow et al., 2012).

### Crimean–Congo hemorrhagic fever virus (CCHFV)

CCHFV (genus *Nairovirus*, family *Bunyaviridae*) is an enveloped single-stranded negative sense RNA virus with a tri-segmented genome characterized by a great genetic variability with complex evolutionary patterns (Anagnostou and Papa, 2009). The recent emergence of CCHFV causing either sporadic human infections or epidemics in previously unaffected areas has raised animal and public health concerns (Papa et al., 2015).

CCHF has been described over a wide geographic area and it is endemic in focal areas in Asia, Europe and Africa. The natural vector and reservoir has been identified as *Hyalomma* spp. ticks, and the geographic distribution of human cases closely mirrors the vector distribution. The virus can be also transmitted to humans by direct contact with blood or tissues of viremic patients or animals. Nosocomial and intrafamily transmission have been reported as well (Aradaib et al., 2010; Papa et al., 2015). CCHFV circulates in nature in unnoticed enzootic tick–vertebrate–tick cycles (Spengler et al., 2016). Thus, a critical transmission rate of CCHFV is reached when adequate density of reservoir hosts is established together with the presence and abundance of the tick

vectors (Estrada-Peña et al., 2012a; Hartemink et al., 2008). Respectively, a modelling study revealed that the increased abundance of large hosts (e.g. wild and domestic ungulates) on which adult ticks feed allowing further amplification through transovarial transmission, is the most important factor for increased transmission of CCHFV (Estrada-Peña et al., 2012b).

Clinical disease, characterized by fever and hemorrhagic manifestations, is restricted to humans and is fatal in 3%–30% of cases. Though, asymptomatic CCHFV infection has been reported in numerous wild and domestic animals (Nalca and Whitehouse, 2007; Spengler et al., 2016). Cattle, sheep, goats, horses, pigs, dogs, and chickens are among the most investigated domestic animals internationally with cattle (79.1% in Afghanistan), sheep (75% in Afghanistan) (Mustafa et al., 2011), goats (66% in Turkey) (Tuncer et al., 2014), horses (58.8% in Iraq) (Tantawi et al., 1981) reaching particularly high seroprevalence in some areas. Domestic animal species are often implicated in CCHFV transmission to humans; sheep have been recognized as very important CCHFV reservoirs in certain endemic regions, and have been epidemiologically linked to human cases (Haghdoost et al., 2013; Humolli et al., 2010; Papa et al., 2013; Yen et al., 1985). Increased IgG seropositivity against CCHFV in livestock often parallels reports of CCHF human cases with exposure to livestock (e.g., slaughterers, butchers and farmers) while lack of seropositive domestic animals indicates either low-level transmission or absence of CCHFV in those geographic areas (i.e. Germany, Italy, the Netherlands, Australia, New Zealand) (Spengler et al., 2016).

Among the wild animals investigated, considerable seroprevalence was reported in hares (3%–22%), buffalo (10%–20%), and rhinoceroses (40%–68%). Data on European brown hare comes from Russia (20%) and Hungary (6%) (Hoogstraal, 1979; Németh et al., 2013). Though, seropositivity has been also reported in *L. capensis* in Zimbabwe (22.6%) and Turkmenistan, *Lepus* spp in Zimbabwe (14.3%), Bulgaria (3%) and Iran and *L. saxatilis* in Zimbabwe (14.5%) (Hoogstraal, 1979; Shepherd et al., 1987; Spengler et al., 2016). Reports of CCHFV isolation from animals are limited to cattle (Kenya and Nigeria), goat (Nigeria and Senegal), hedgehog (Nigeria) and European brown hares (Crimea). This scarcity of isolates from animals together with the data obtained from experimental studies, likely reflect a relatively brief viremic period and difficulty in identifying infected animals due to absent or mild clinical disease (Fagbami et al., 1975;

Shepherd et al., 1989; Smirnova, 1979) instead of lack of infection in these animals. Although some species may serve as direct sources of viral transmission (e.g., viremic livestock) the role of hares in the epidemiology of CCHFV stems for the fact that this species aid principally in maintaining high levels of CCHFV endemicity (Spengler et al., 2016).

### Hepatitis E virus (HEV)

Hepatitis E virus is a small non-enveloped single-stranded positive sense RNA virus with an icosahedral capsid (Meng, 2010), belonging to the genus *Orthohepevirus* within the family *Hepeviridae* (Pavio et al., 2015). The genus *Orthohepevirus* contains four species designated as *Orthohepevirus* A to D. *Orthohepevirus* A contains seven genotypes (HEV-1 to HEV-7) and a putative new genotype 8 that can infect humans and/or a wide variety of mammals (Smith et al., 2014; Woo et al., 2014) (Lee et al., 2016; Smith et al., 2014; Woo et al., 2016).

HEV-1 and HEV-2 are mainly restricted to humans and have been responsible for large waterborne epidemics of hepatitis E. However, several primate species have been also shown to be susceptible for HEV-1 and HEV-2 (Pavio et al., 2015; Spahr et al., 2017). HEV-3 and HEV-4 have been detected in both, humans and animals, and are the main cause of sporadic cases of hepatitis E in many industrialized countries (Pavio et al., 2015). Domestic pig (*Sus scrofa domesticus*) and wild boar (*Sus scrofa*) are considered to be the main animal reservoirs for HEV-3 and HEV-4 (Caruso et al., 2017; Johne et al., 2014). Although deer to human transmission has been reported repeatedly, deer is not regarded as a true HEV reservoir as this species probably undergoes spillover infections from wild boar (Anheyer-Behmenburg et al., 2017). A distinct subtype of HEV-3 has been repeatedly detected in rabbits (*Oryctolagus cuniculus*) and was recently also identified in a few human patients (Abravanel et al., 2017; Spahr et al., 2017).

Evidence for transmission of HEV-3 and HEV-4 by direct contact of humans with animals has been described. Persons with professional exposure to domestic pigs and wild boars such as slaughterers, pig farmers, forestry workers, hunters or veterinarians exhibit significant higher anti-HEV antibody prevalences than the general population (Dremsek et al., 2012; Meng et al., 2002; Pavio et al., 2015; Schielke et al., 2015). Moreover, foodborne infections with HEV-3 and HEV-4 due to consumption of

undercooked meat and meat products derived from infected pigs, wild boars and deer have been repeatedly reported. HEV can be transmitted by milk consumption while other types of food like berries and shellfish have been suspected to act as means for HEV transmission after contamination with animal faeces (Pavio et al., 2015; Spahr et al., 2017). Transmission of HEV-3 has also been described by parenteral routes due to blood tranfusion or organ transplantation (Abravanel et al., 2017; Kamar et al., 2013, 2008).

Several studies conducted in European and Asian countries revealed the presence of HEV antibodies or HEV RNA in domestic and wild animals including domestic pig (*Sus scrofa domestica*), wild boar (*Sus scrofa*), red deer (*Cervus elaphus*), fallow deer (*Dama dama*), roe deer (*Capreolus capreolus*), sika deer (*Cervus nippon*) and wild rabbit (*Oryctolagus cuniculus*) (Spahr et al., 2017). Recently, seroprevalence of 2.2% was reported in European brown hare in Germany while HEV RNA was not detected in any of the samples examined. In the same study, a seroprevalence of 37.3% was observed in wild rabbits and 17.1% of the samples were HEV RNA positive. This low seroprevalence in hares is indicative of their assumed minor importance in the epidemiology of HEV in Germany while the difference between the two species may be attributed to their different habitats and biological behaviours; European brown hares live in open fields, while wild rabbits live in their own burrows with increased risk for transferring pathogens to each other due to closer social contacts (Hammerschmidt et al., 2017). Previous studies in European brown hare in Italy provided no evidence of antibodies against HEV (Mazzei and Forzan, 2015) or HEV RNA in hares (Serracca et al., 2015).

### Pestiviruses

Pestiviruses are enveloped single-stranded RNA viruses of positive polarity that belong to the family *Flaviviridae*. The genus *Pestivirus* nowadays comprises of four species, classical swine fever virus (CSFV), bovine viral diarrhea virus types 1 and 2 (BVDV-1 and BVDV-2) and border disease virus (BDV) of sheep as well as several exotic viruses. Further molecular characterization of new genus members has led to the proposal of a classification with eight pestivirus species which is under discussion by the responsible ITCV study group (King et al., 2011; Tautz et al., 2015).

Pestiviruses are shed in all body secretions of infected animals. Transmission can occur directly by contact between animals or indirectly via contact with infectious secretions, contaminated food, or needles while transmission via semen is another important root (Chae and Choi, 2002; Meyling et al., 1990). In pregnant animals, all pestiviruses can also be transmitted vertically by crossing the placenta and infecting the fetus (Tautz et al., 2015).

Despite considerable efforts such as stamping-out strategies, vaccination and strict biosafety measures, pestiviruses continue to cause severe losses thus being among the economically most important pathogens of livestock (Lindenbach et al., 2013). Even in countries that managed to eradicate a given pestivirus, the risk of reintroduction represents a constant threat. The high frequency of transport and trade of animals susceptible to pestivirus infection and the specific survival strategies gained during evolution such as their ability to establish long-lasting persistent infection in their host animals, are among the most important difficulties to control pestiviruses (Lindenbach et al., 2013; Tautz et al., 2015).

Antibodies against BVDV have been detected in a wide range of wild and domesticated ruminant and porcine species (Becher et al., 1997; Grant et al., 2015; Scherer et al., 2001; Van Campen et al., 2001) while persistent infection has been documented in sheep, goats, pigs, alpaca, white-tailed deer, eland, mouse deer and American mountain goats (Grant et al., 2015).The importance of sympatric wildlife has been widely studied (Vilček and Nettleton, 2006) and the necessity for consideration of wild hosts in epidemiological studies of pestiviruses and especially for the implementation of eradication plans has been suggested (Ståhl and Alenius, 2012). Rabbit is the only non-artiodactyl animal in which virus could be propagated upon intravenous inoculation. More recently, a serological survey in Germany showed that 40% of wild rabbits exhibited low neutralizing antibody titres against BVDV (Frölich and Jürgen Streich, 1998). However, no virus could be isolated from any rabbit. A recent experimental study has demonstrated that rabbits can be infected with BVDV by both parenteral and natural routes but shedding of virus was not detected (Bachofen et al., 2014). Thus, there are indications that rabbits could be a natural wildlife reservoir for BVDV and given their abundance in several countries, often living on or near livestock

pastures, a BVDV reservoir in rabbits could have significant consequences for BVDV eradication campaigns in these countries.

Recently, a study revealed the presence of neutralizing antibodies against ruminant pestiviruses in 36.2% of European brown hares and significant differences between BDV-4 and BVDV-1 titres were found in seven hares, suggesting the inclusion of the European brown hare as the third free-ranging non-artiodactyls species being susceptible to pestivirus infection (Colom-Cadena et al., 2016). However, similarly to previous studies from free-ranging lagomorphs, molecular analyses of the sera from the European hares did not detect Pestivirus RNA (Colom-Cadena et al., 2016; Frölich and Jürgen Streich, 1998). The authors suggested that the high seroprevalence observed in that study could be related to a higher pestivirus circulation in the study areas as Pyrenean chamois populations of this alpine area show high prevalence of antibodies against BDV strongly suggesting a sylvatic cycle and a BDV circulation (Marco et al., 2009). Moreover, a high prevalence of antibodies against pestiviruses in cattle and sheep has been previously reported in the area which along with the high reported densities of domestic ruminants in the alpine pastures represents a chance for virus maintenance (Colom-Cadena et al., 2016).

## Bacterial pathogens

### *Yersinia* spp

*Yersinia pseudotuberculosis* (*Y. pseudotuberculosis*) is a Gram-negative bacillus, non-spore forming, non-lactose fermenter, facultative anaerobe, catalase-positive and oxidase-negative belonging to the family *Enterobacteriaceae* which is classified into serotypes O:1–O:15 and 10 subgroups (O:1a–c, O:2a–c, O:4a–b, and O:5a–b) (Bogdanovich et al., 2003). It has a worldwide distribution in humans and wild and domestic animals. *Y. pseudotuberculosis* has been isolated from carnivorous, herbivorous and omnivorous animals and it is carried subclinically by a range of animal species, including wild mammals, birds and rodents (Najdenski and Speck, 2012). A difference in the worldwide distribution of *Y. pseudotuberculosis* serotypes in humans has been observed as the isolates belonging to the O:1a and O:1b serotypes commonly cause gastroenteritis in humans in Europe and those belonging to the O:1b, O:2b, O:4b

and O:5b serotypes commonly infect humans in the Far East (Fukushima et al., 2001, 2011; Yoshino et al., 1995). The same holds true for animals; strains O:6–O:14 have been only isolated from wild birds and from the environment in the Far East (Fukushima et al., 2001).

Despite its importance in human and animal health as a pathogen and zoonotic agent, the epidemiological features of yersiniosis are still under investigation. The principal reservoirs of *Y. pseudotuberculosis* are rodents and birds while hares, common voles (*Microtus arvalis*) and water voles (*Arvicola terrestris*) are also known to serve as reservoirs (Najdenski and Speck, 2012). Recent surveys suggest that pigs are the primary reservoir of human pathogenic *Yersinia enterocolitica* (*Y. enterocolitica*) and, to a lesser extent, of *Y. pseudotuberculosis* in Europe (Bonardi et al., 2016; Vanantwerpen et al., 2014).

Fecal-oral transmission is the main route of natural *Y. pseudotuberculosis* infection. The agent is found ubiquitously in the environment where it can survive for a long time. The environment itself is contaminated from the faeces of infected animals, mainly rodents and birds (Najdenski and Speck, 2012). *Y. pseudotuberculosis* has been isolated from fresh water such as river, well, and mountain stream (Fukushima, 1992; Fukushima et al., 1995). Ingestion of environmental contaminated substances at pasture or watering places and preying upon infected animals are considered important routes of transmission (Fratini et al., 2017; Najdenski and Speck, 2012). The contact between livestock and wildlife is the most important factor in disease transmission (Bengis et al., 2002). As for alternative routes of transmission, the role of insect vectors needs further elucidation, milk - borne spread is possible in cases of yersinial mastitis, venereal transmission through semen is possible, transplacental spread to the fetus has been recorded in several species and vertical transmission is also a possibility (Hubbert, 1972; Najdenski and Speck, 2012). Human infection with *Y. pseudotuberculosis* and *Y. enterocolitica* is acquired by direct or indirect contact with domestic animals, wild animals, birds or consumption of contaminated food and water (Najdenski and Speck, 2012).

The disease occurs mainly during winter when animals, particularly free – living species, are exposed to cold and starvation. Wet land areas are prone to keep the disease in a steady state throughout year. Cold and humid weather, stress, shortage of food supply

and endoparasitic infections are considered predisposing factors. Despite its worldwide distribution, the disease presents higher prevalence in temperate climates and is more frequent in winter (December – March) than summer (Najdenski and Speck, 2012; Wibbelt and Frölich, 2005).

Literature on yersiniosis in animals is scarce and restricted to case reports. The most common clinical feature caused by *Y. pseudotuberculosis* in wild and domestic animals is subclinical enteric disease, but abortions in cattle (Hannam, 1993), sheep (Otter, 1996) and goats (Witte et al., 1985) have been also reported. A septicaemic form, called pseudotuberculosis, can occur in lagomorphs, rodents, including laboratory rodents, and caged birds (Fratini et al., 2017; Wobeser et al., 2009). Although the term pseudotuberculosis is originally referred only to infection with *Y. pseudotuberculosis*, the fact that several strains of *Y. enterocolitica* can mimic the clinical manifestations of pseudotuberculosis in hares, has led to the inclusion of *Y. enterocolitica* infections in the pseudotuberculosis syndrome (Frandölich et al., 2003).

Pseudotuberculosis is a typical disease of lagomorphs as it is commonly diagnosed in hares and it constitutes one of the most important and frequent causes of death in hare populations with losses of up to 50% (Frandölich et al., 2003; Wobeser et al., 2009). It is estimated that every hare will have been exposed to *Y. pseudotuberculosis* at some point during its life time (Frandölich et al., 2003). The course of disease is acute to chronic with dyspnoea and diarrhoea. Clinical signs include mild to severe enteritis, enlargement of the spleen and various lymph nodes (Frandölich et al., 2003). Post mortem examination of infected subjects reveals granulomatous nodules in several organs, multifocal caeseous necroses in spleen, liver, intestine and mesenteric lymph nodes (Frandölich et al., 2003; Najdenski and Speck, 2012; Wobeser et al., 2009).

A study performed in Germany on 230 European brown hares revealed a seroprevalence of 89.6% for *Y. pseudotuberculosis* and *Y. enterocolitica* (Bartling et al., 2004). In another study, antibodies against *Yersinia* spp. were found in 55% of European brown hares examined. In contrast, studies in other parts of Germany reported seroprevalences ranging from 13–17% (Frandölich et al., 2003). It has been proposed that this difference may have been attributed to failure to diagnose *Y. enterocolitica* infected animals due to the use of laboratory techniques based on Lipopolysaccharide (LPS) antigen and a possible increase of *Yersinia* infection in European brown hares

(Frandölich et al., 2003). *Y. pseudotuberculosis* was isolated from 13% of hares found dead in Schleswig-Holstein, Germany whereas *Y. enterocolitica* was isolated from only 4% of these hares (Wuthe et al., 1995). In a study conducted recently in Italy, animal *Y. pseudotuberculosis* isolates were characterized for the O-genotype and the majority of hare isolates belonged to O:1a and O:1b serotypes which is common for isolates collected from Western European countries. Besides, strains belonging to O:1 serotype are the most common cause of yersiniosis in humans, followed by strains belonging to the O:2 and O:3 serotypes (Chiesa et al., 1993; Wunderink et al., 2014). However, O:2a serotype was also detected in hares and interestingly, serotype O:12–O:13 was found in a hare from Northern Italy; this was the first repot of this particular serotype in Europe. The introduction of hares from Eastern European countries during repopulation or the introduction of stains by migratory birds, were suggested by the authors as possible explanations for this finding, highlighting the possible introduction of *Y. pseudotuberculosis* strains in Italy from Far Eastern countries in the past (Magistrali et al., 2015).

### *Brucella* spp

*Brucella* spp. are facultative intracellular gram-negative cocco-bacilli, non-spore-forming and non-capsulated. Infections by *Brucellae* have been found worldwide in a great variety of terrestrial domestic and wildlife species as well as a wide variety of marine mammals (Godfroid, 2012; Seleem et al., 2010). Nine *Brucella* species are currently recognized; *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, *B. neotomae*, and *B. microti* (Scholz et al., 2008; Verger et al., 1987) affecting terrestrial animals and *B. ceti* and *B. pinnipedialis* that affect marine mammals (Foster et al., 2007). The zoonotic nature of *Brucella* spp is well known for *B. melitensis,* the most pathogenic and invasive species for human, followed by *B. suis*, *B. abortus* and *B. canis* while the zoonotic potential of *B. ceti* has been also documented (Seleem et al., 2010).

The consumption of aborted fetuses and fetal membranes or contaminated food is an important route of transmission in animals. However, the bacteria may also spread through venereal, conjunctival–mucosal and transplacental routes (European Food Safety Authority (EFSA), 2009). Regarding *Brucella* infection in humans, in brucellosis endemic regions, infection may be acquired via contact with infected animals or

consumption of their products, mostly milk and milk products (e.g. cheese made from unpasteurized milk of sheep and goats and rennet from infected lambs and kids). Farm workers, veterinarians, ranchers, and meat-packing employees are considered at higher risk and brucellosis is referred as an occupational disease for these groups (Tabak et al., 2008). Person-to-person transmission occurs rarely. Consequently, as it has been shown previously, eradication of the disease from the natural animal reservoirs leads to a dramatic decrease in the incidence of human infection (Cook et al., 2002; Seleem et al., 2010).

*Brucella* infection in wild animals is not always the result of pathogen spillover from domestic animals to wildlife but it may represent a sustainable infection in wild animals (Godfroid, 2012). The presence of *Brucella* carriers among wild animals is an established fact; hares, wild reindeer, bison and some rodent species carry *Brucella* spp regardless of their prevalence among the main hosts (Zheludkov and Tsirelson, 2010). As for hares, this species has been shown to be infected by *B. suis* biovars 1 and 2 (Fort et al., 2012) and along with wild boars (*Sus scrofa*), hares are an important reservoir of *B. suis* biovar 2 (Godfroid, 2012). Biovar 1 is highly pathogenic and cause severe disease in humans while biovar 2 is rarely pathogenic or non-pathogenic to humans and has only exceptionally been described as the causative agent of human brucellosis (Paton et al., 2001; Teyssou et al., 1989). However, the importance of *B. suis* biovar 2 stems for the fact that it can infect domestic pigs and even cattle (EFSA, 2009). Thus, hares and wild boars may serve as a source of infection for free ranging pigs and cattle due to cross border and long distance movements through migration of wild boars or live animal translocations of hares (Godfroid, 2012; Kreizinger et al., 2014). Moreover, dead hares can apparently be the source of infection for wild and domestic animals and birds of prey (Zheludkov and Tsirelson, 2010).

The role of hares in the dissemination of brucellosis is probably of lesser importance than that played by wild boar. Data on the geographical distribution of *Brucella* infection in European brown hare, although limited compared to wild boar, are suggestive of a less important prevalence in this species. The species ecology, that is a non-migratory way of life and occupation of small home ranges, most probably leads to a patchy pattern of the foci of infections and to a reduced interface with other wildlife (Godfroid et al., 2013). Occurrence of *Brucella* in free-ranging hares reflects

their previous or current contact with infected wild boars or infected domestic pigs (Frandölich et al., 2003). Thereafter, hares can maintain *B. suis* biovar 2 and infect domestic animals (grazing pigs and cows) even in the absence of a wild boar population (Godfroid et al., 2005). In fact, between 1929 and 1999, ten clinical outbreaks of *B. suis* biovar 2 in domestic pigs in Denmark, were linked to hares (Godfroid et al., 2013). During five *Brucella* epizootics in pigs in Denmark, approximately 4500 hares were examined, and more than 3% were *B. suis* positive. Interestingly, the areas of epizootics among domestic pigs, coincided with the foci of hare brucellosis (Zheludkov and Tsirelson, 2010). Hares were also considered as a possible source of *B. suis* biovar 2 outbreaks in domestic pigs via swill feeding with offal from hunted infected hares. This species has been deemed as the source of *B. suis* biovar 2 infections in cattle in Denmark, since there is no established population of free-ranging wild boar in the area (Godfroid et al., 2013). Other outbreaks have been attributed to the introduction of infected animals from holdings where the disease had not been detected (EFSA, 2009). Hares and rabbits are relatively resistant to *B. melitensis* infection. Therefore, it seems more probable that farm animals acquire infection from hares than the opposite. Such cases were reported from Latvia where two *B. abortus* strains were isolated from 10 dead hares with postmortem findings indicative of brucellosis (Zheludkov and Tsirelson, 2010). A study conducted recently, showed that European *B. suis* strains of hare origin were closely related to one another and they did not cluster according to their geographic origin. The results of this study were suggestive of cross-border live animal translocation and of certain *B. suis* strains adaptation to hares. European isolates from domestic pigs were closely related to isolates of hare and wild boar origin, indicating that these species are a source of brucellosis in domestic pigs (Kreizinger et al., 2014)

Reported seroprevalences in hares range from 0% to 17%. In Lower Austria *Brucella* sp. were detected in 2.7% of hares, with higher rates in the northeastern districts and in Styria, *Brucella* sp. was isolated from 4.5% of European hares (Winkelmayer et al., 2005).

The infection in hares is either latent or it is characterized by inflammation and abscesses in the reproductive system or miliary abscesses in the lymph nodes, liver, spleen, kidneys, urinary bladder, joints and brain (Godfroid et al., 2005; M. Gyuranecz et al., 2011; Kreizinger et al., 2014). Brucellosis was reported to be more prevalent in

adult hares compared to subadult ones which may be attributed to its chronic nature and the spread during reproduction (Treml et al., 2007). In general, it seems that this pathogen contributes only little to the overall mortality of hares (Winkelmayer et al., 2005).

Natural foci of brucellosis seem to be independent of the hare population density which can be hypothetically explained by the so-called small home range and a sufficiently low threshold of the disease in this species (Kunst et al., 2001; Pikula et al., 2005). Other factors such as climatic factors that influence the survival of the pathogen in the environment and the chronicity of the disease may affect the maintenance of natural foci of brucellosis (Pikula et al., 2005). The disease may exist in the hare environment thus constituting a threat of contracting the pathogen, independently of the hare population density, or the territory is brucellosis-free. In the latter case, animal translocation due to re-populating efforts in hunting grounds or migration of the wild boar, can bring *Brucella* spp in a brucellosis-free territories (Pikula et al., 2005).

### *Francisella tularensis*

*Francisella tularensis* is a Gram-negative, non-motile, non-sporing, aerobic coccobacillus (Sjöstedt, 2007) which is divided into four sub species; *F. tularensis* subsp. *tularensis* (type A strains), *F. tularensis* subsp. *holarctica* (type B strains), *F. tularensis* subsp. *mediasiatica*, and *F. tularensis* subsp. *novicida*. The sub species *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* are the main cause of tularaemia worldwide (Sjöstedt, 2007). Although a few type A strains have been isolated from environmental sources and arthropods in Slovakia and Austria, they are mainly found in North America (Chaudhuri et al., 2007). They are considered the most virulent strains, classified in the World Health Organization (WHO) Risk Group 3. The infectious dose is very low; 10–50 bacteria inhaled or injected intradermally can reliably cause disease in humans (Richard and Oppliger, 2015). The causative agent of tularaemia in Europe is type B strains (Maurin and Gyuranecz, 2016). These strains are reported throughout the northern hemisphere (Sjöstedt, 2007), but isolates have also been identified in Tasmania (Jackson et al., 2012). The distribution of *F. tularensis* subsp. *mediasiatica* is restricted to Central Asia whereas *F. tularensis* subsp. *novicida* have

been associated with waterborne transmission in Australia and the USA (Gyuranecz, 2012).

*F. tularensis* has a remarkably broad host range and complex epidemiology. Natural *F. tularensis* infection has been reported in a wide range of vertebrates, including mammals, birds, amphibians and fish, and in certain invertebrates (Gyuranecz, 2012). The disease primarily concerns the genera *Lagomorpha* and *Rodentia*. The European brown hare (*Lepus europaeus*) is a common host of tularaemia in Central Europe and this species is moderately sensitive to *F. tularensis* infection. On the other hand, the disease occurs frequently in mountain hares (*Lepus timidus*) in Scandinavia and Russia and it is often fatal. Thus, European brown hare is regarded as a reservoir due to its possible capability to maintain the pathogen for a longer period than the mountain hare (Gyuranecz et al., 2010). Rodents play an important role in the maintenance of enzootic foci in Eurasia while voles are most frequently involved in tularaemia epizootics. In fact, the water vole (*Arvicola amphibius*) and the common vole (*Microtus arvalis*) are highly susceptible to the disease and they may also present chronic infection, thereby acting as reservoirs the periods in between the epizootics (Gyuranecz, 2012). Though, since small rodents and lagomorphs often develop fatal infections, the existence of alternative unidentified reservoirs such as domestic animals, able to transmit the disease to humans, is also possible (Weinberg and Branda, 2010). Besides, although infection is rare among domestic animals, outbreaks can occasionally occur among sheep during lambing season (Gyuranecz et al., 2011). Haematophagous arthropods, especially ticks, serve as mechanical as well as biological vectors; they enable amplification of the bacteria thus contributing to re – transmission, and they maintain the bacterium throughout its multiple life stages (Gyuranecz, 2012).

The two known transmission cycles of tularaemia are the terrestrial and the aquatic cycle. In the terrestrial cycle, hares and rodents are the most important mammalian hosts contaminating the environment through their body discharges, whereas haematophagous arthropods serve as vectors (Gyuranecz, 2012). Other routes of transmission that have been described in hares in Europe include aerogenous infection (Gyuranecz et al., 2010) and the alimentary route in mountain hares in Scandinavia (Mörner et al., 1988). In the aquatic cycle, voles and possibly muskrats and beavers

serve as the main host species. Water contamination could be maintained by the faecal matter of infected animals, or by infected animal carcasses (Gyuranecz, 2012).

The major modes of *F. tularensis* transmission to humans include direct transmission from the animal reservoir, arthropod bites, and transmission through contaminated water and soil. Human tularaemia cases from aquatic sources are more common and often occur as large outbreaks (Maurin and Gyuranecz, 2016). Direct transmission through handling the meat and fur of an infected animal, mainly a brown hare, might also occur since the bacterium is capable of penetrating healthy skin (Richard and Oppliger, 2015). This is the main mode of human infection in Central Europe (Keim et al., 2007; Richard and Oppliger, 2015). Ingestion of undercooked meat prepared from an infected animal, or animal bites (especially from small rodents, cats, and dogs) represent an additional danger (Keim et al., 2007; Richard and Oppliger, 2015). Arthropod-borne transmission is common in the USA after a tick bite, a mosquito bite, or, rarely, bites from other arthropods (Richard and Oppliger, 2015). *F. tularensis* can persist for weeks to months in the environment. Thus, inhalation of contaminated dusts aerosolized from soil, faecal matter and dead animals, contact or ingestion of contaminated water (e.g. drinking water from tanks and wells) are other routes of transmission (Maurin and Gyuranecz, 2016; Richard and Oppliger, 2015).

In a study conducted recently in Hungary, the factors influencing the emergence of Tularemia were investigated and the authors suggested that the number of tularemia cases in humans was positively associated with the seroprevalence of *F. tularensis* among European brown hares and the population density of common voles (Gyuranecz et al., 2012). The high vole population density leads to increased transmission and spillover to hares by stress-related aggression, cannibalism and contamination of the environment by infectious body discharges. This enhanced transmission and spillover may lead to epizootics and the spread of the disease may be further facilitated by the bacterial shedding in the urine of the infected hares (Gyuranecz et al., 2012, 2010). It becomes clear that the presence of a high number of sources of infection may lead to increased human tularaemia cases via the handling and skinning of hares, and, potentially, the inhalation of infectious aerosols. Moreover, as rodents and lagomorphs are hosts of blood sucking arthropods, a high number of infected hosts may lead to an increased number of infected ticks and thereby, increased transmission of the

pathogen to humans. Interestingly, the hare population density was negatively correlated to the seroprevalence of *F. tularensis* in hares. Besides, the disease in hares is often fatal causing septicemia and death, thus decreasing the population density of this species (Gyuranecz et al., 2012, 2010).

In moderately susceptible species such as the European brown hare, the disease can be subacute. In the late stages of the disease, the clinical manifestations include depression, stupor, loss of body weight and lack of fear, facilitating capture. At necropsy, numerous, randomly distributed, well - demarcated, greyish - white or yellowish - white foci, can be observed most frequently in the lungs, the pericardia and the kidneys and sometimes in the testicles, bone marrow and mammary glands (Gyuranecz et al., 2010). A recent study revealed that subpopulations of *F. tularensis* subsp. *holarctica* (clade B.13 and B.FTNF002-00) may be associated with different pathologic findings in the European brown hare. In fact, in natural infection with *F. tularensis subsp. holarctica* (clade B.13), the most commonly affected tissues are lung, pericardium, and kidney (Gyuranecz et al., 2010). On the other hand, strains of the clade B.FTNF002-00 were almost invariably associated with splenitis and hepatitis (Origgi and Pilo, 2016). Some authors consider the European brown hare highly susceptible to *F. tularensis* and that marked bacteraemia may be caused by even extremely low infectious doses of the bacterium. Thus, infected hares may be an important source of infection for blood sucking arthropods and their carcasses and excrements may contaminate the environment. A chronic form of the disease has been described for those individuals that survive infection being a permanent source of infection for other animals sharing the same living space as well as for humans (Treml et al., 2007). Hares seroconvert, and potentially they can carry viable bacteria over a longer time span and thus serve as a reservoir species (Gyuranecz et al., 2010).

Previous studies in other European countries showed that the seroprevalence of *F. tularensis* in hares range from 0% (Frandölich et al., 2003) to 7% (Winkelmayer et al., 2005). In Germany, during 2006-2009, the DNA prevalence of *F.tularensis* in European Brown hare was 1.1%. Importantly, the prevalence was higher for the hares found dead compared to the hunted ones (2.9% and 0.7% respectively) suggesting that hares became seriously diseased following infection and died (Runge et al., 2011).

## Protozoal pathogens

### *Toxoplasma gondii*

*Toxoplasma gondii* is a significant zoonotic obligate intracellular parasite with a worldwide distribution that affects humans and warm-blooded animals (Dubey and Dubey, 2010). The only known definitive hosts for *T. gondii*, able to produce and shed the environmentally-resistant stage, oocysts, in their feces are the domestic and free-ranging felids. All warm-blooded animals, including mammals and birds, can act as intermediate hosts of *T. gondii* as the parasite multiplies and encysts in many tissues (Dubey and Jones, 2008). The oocysts are shed by felids, the parasite is introduced in new areas through migrating animals and it is harbored in the tissues of prey animals being able to infect predators and scavengers (Jokelainen, 2012). Climatological conditions such as humidity, rainfall and temperature impact the oocyst survival and sporulation in localized areas. In fact, a positive and significant correlation of antibody prevalence of *T. gondii* between locations and rainfall has been observed in wildlife species in Spain (Gamarra et al., 2008).

Humans become infected post-natally by ingesting tissue cysts from undercooked meat, consuming food or drink water contaminated with oocysts, or by accidentally ingesting oocysts from the environment. Consumption of raw or almost raw, dried, cured or smoked meat from domestic animals, unpasteurized goat milk or consumption of meat from wild animals may be associated with ingestion of the parasite (Bartova and Sedlak, 2012). Humans, especially hunters, may also get infected through contact with the parasite while dressing game (Dubey and Dubey, 2010; Fernández-Aguilar et al., 2013). Frequent contact with animals and soil, such as in the case of abattoir workers, garbage handlers and waste pickers was recognized as a factor associated with higher *T. gondii* prevalence (Dubey and Beattie, 1988). Moreover, infection by direct contact was reported for children playing with dogs and cats as animals can act as mechanical vectors (Etheredge et al., 2004). However, only a small percentage of exposed adult humans develop clinical signs of disease and whether the severity of toxoplasmosis in immunocompetent hosts is due to the parasite strain, host variability or other factors is still under investigation. In fact, recently, attention has been focused on genetic variability among *T. gondii* isolates from apparently healthy and sick hosts (Dubey and Jones, 2008).

*T. gondii* can cause disease in various hosts and it is a frequent cause of early embryonic death and resorption, fetal death and mummification, abortion, still birth and neonatal death in animals. Thus, toxoplasmosis in domestic and farm animals is a disease of great importance for veterinary medicine and husbandry since it can cause productive and economic losses (Bartova and Sedlak, 2012). Regarding hares, they are considered as exceptionally susceptible to primary infection (Gustafsson et al., 1997; Lindsay and Dubey, 2014). Fatal toxoplasmosis has been repeatedly described in hares (Christiansen and Siim, 1951; Gustafsson et al., 1997) and it is frequently found in hare populations (Frölich et al., 2003b). Typically, toxoplasmosis in hares is acute and they are in a normal nutritional state (Christiansen and Siim, 1951; Gustafsson et al., 1988). Interstitial pneumonia, multifocal areas of hepatocellular necrosis, encephalitis, and moderate necrosis of lymphoid follicles of the lymph nodes are the predominant lesions in hares in endemic areas (Christiansen and Siim, 1951; Gustafsson, 1997; Gustafsson et al., 1997).

It has been suggested that stress or environmental factors like winter and harsh climatologic conditions, may trigger acute fatal toxoplasmosis in hares (Sedlák et al., 2000). Besides, most of the fatal toxoplasmosis cases have been reported from Northern countries of Europe, where harsh winter conditions are common. For instance, in a study conducted previously in the Czech Republic, all the hares that died during the experiment, were kept in outdoor boxes in temperatures below 0 °C (Sedlák et al., 2000). In Denmark and Sweden, most of the fatal cases were observed in animals sampled during the cold months. However, an outbreak in Japan occurred during the warm season (Christiansen and Siim, 1951; Shimizu, 1958). Hares get infected through ingestion of food or water contaminated with oocysts from domestic cat or free-ranging felid feces with which they share the same habitats (Lindsay and Dubey, 2014). Infected hares can act as a potential source of *T. gondii* for other animals, especially for carnivores but also for humans (Bártová et al., 2010).

Experimental infections have demonstrated that toxoplasmosis in hares differs from that in rabbits (*Oryctolagus cuniculus*). In a previous study, postmortem examination one week after inoculation with 50 oocysts of a local *T. gondii* isolate in mountain hares, revealed the presence of parasites in the majority of the tissues examined and extensive damage. Only minor lesions were observed in rabbits. No differences in the

production of antibodies were observed, but the mountain hares seemed to lack a proper cellular response (Gustafsson, 1997; Gustafsson and Uggla, 1994). In another study, 12 brown hares were inoculated with 10, 1,000, or 10,000 oocysts and all of them died, whereas, only two out of the 12 rabbits, suffering from a concurrent accidental *Pasteurella* infection, finally died (Sedlák et al., 2000). *T. gondii* was isolated from liver, brain, spleen, kidney, lung, heart and skeletal muscles (Sedlák et al., 2000).

The proposed natural, inherent susceptibility of hares to *T. gondii* is well supported by cases of fatal toxoplasmosis, with a concomitant low prevalence of latent chronic or subclinical infections (Christiansen and Siim, 1951; Jokelainen et al., 2011). In contrast, there are other studies that reported high *T. gondii* seroprevalence in European brown hares (Frölich et al., 2003b), suggesting they can survive *T. gondii* infection in the wild. The explanations for the failure to achieve equilibrium between the host and the parasite are controversial and focus on the characteristics of the host (Gustafsson et al., 1997; Maubon et al., 2008; Sedlák et al., 2000).

*T. gondii* antibodies have been found in animals worldwide and although for most host species, the seroprevalence numbers are clearly higher than incidence of clinical and fatal cases, European brown hare appears exceptionally susceptible to the infection (Jokelainen et al., 2011). Seroprevalence of *T. gondii* in hares varies among countries ranging from 0-46%. The highest seroprevalence against *T.gondii* (46%) was found in hares from Germany by the Sabin-Feldman test (Frölich et al., 2003b). In Slovakia, 32% prevalence was found by complement fixation test (CFT) (Catar, 1972). IFAT was used by Bartova and Sedlak, 2012 and Edelhofer et al., 1989 in Czech Republic and Austria reporting a seroprevalence of 20% and 2% respectively. In France 9% of the hunted brown hares were antibody-positive by modified agglutination test (MAT) but isolation attempts were unsuccessful (Aubert et al., 2010). Poli et al., 1987 reported low prevalence (6.4%) in Tuscany (Italy) by indirect agglutination test. No antibodies were detected in hares from Sweden using IFAT, ELISA, DAT and Sabin-Feldman test (Gustafsson and Uggla, 1994). Although different serological techniques were used in the above-mentioned studies, the differences in the serological status of hare population against *T. gondii* across European countries are indeed highlighted. Besides, *T. gondii* seroprevalence varies even within different areas of a country and within the same city (Jokelainen et al., 2011). In a previous study in Spain, significantly higher

seroprevalence was observed in juvenile Iberian hares compared to the adult ones (Fernández-Aguilar et al., 2013). The authors suggested that possibly *T. gondii* infection affects the survival of infected hares and thus infected juveniles reach the adult stage at lower rates than the non-infected hares or that a short-lived humoral immune response against *T. gondii* exist in Iberian hares (Fernández-Aguilar et al., 2013). Nevertheless, the dynamics of *T. gondii* antibodies in hares is not well understood as it has only been analyzed in short term experimental infections because of hares suffering or dying following infection (Gustafsson, 1997; Sedlák et al., 2000).

Regarding fatal toxoplasmosis cases in hares, in Sweden, toxoplasmosis was the cause of death in 10% of European brown hares and 4% of mountain hares necropsied in the 1980s (Gustafsson et al., 1988). In Denmark a similar proportional mortality rate of almost 10% in brown hares was also reported earlier (Christiansen and Siim, 1951). In Germany, 57% of the hares examined were immunohistochemically positive for *T. gondii* antigen (Frölich et al., 2003b). In addition to these findings from wild hares, an outbreak of acute toxoplasmosis with high mortality was described on a mountain hare ranch in Japan (Shimizu, 1958).

### *Leishmania spp*

Protozoan parasites of the genus *Leishmania* (*Trypanosomatida*: *Trypanosomatidae*) are the causative agents of a complex of vector-borne diseases, Leishmaniases, which represent an important public health concern. Approximately 53 *Leishmania* species have been described; of these, 31 species are known mammal parasites and 20 species are pathogenic for humans. They are present in extremely diverse ecosystems and are able to infect a wide range of mammals (Bañuls et al., 2007). Leishmaniases are endemic in large areas of the tropics, subtropics, and the Mediterranean basin, including more than 98 countries. It is estimated that there is a total of 350 million people at risk and 12 million cases of infection. Canine leishmaniasis is a serious veterinary problem with an estimate of 2.5 million infected dogs in the Mediterranean basin only (Alvar et al., 2012).

The transmission mainly occurs through the bites of infected female sandflies of the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World. Approximately

166 sandfly species have been reported to be proven or potential vectors of different *Leishmania* species in the Old and New World. Among these species, 78 are reported as the proven vectors of *Leishmania*. Among the above-mentioned sandfly vectors, seven are involved in the transmission of *L. major*, seven in the transmission of *L. tropica*, 31 in the transmission of *L. infantum*, and nine in the transmission of *L. donovani* (Akhoundi et al., 2016). *Leishmania* species are heteroxenous as they infect the phagocytes of the reticuloendothelial system of mammals and the intestinal tract of phlebotomine sandflies. However, other arthropods such as *Forcipomyia* spp. (Diptera: *Ceratopogonidae*) as well as some tick species have been reported as the potential vectors of *Leishmania* spp (Dantas-Torres et al., 2010; Slama et al., 2014; Solano-Gallego et al., 2012). Importantly, sexual, vertical and iatrogenic transmission have been also reported (Morillas-Marquez et al., 2002; Pangrazio et al., 2009; Silva et al., 2009).

The successful zoonotic or anthroponotic transmission between the sandfly vector and a mammalian reservoir is a key factor for survival of the *Leishmania* parasite. Zoonotic transmission cycles involve a reservoir host such as dogs in domestic cycles and rodents, marsupials, edentates, monkeys and wild canids in sylvatic cycles where enzootic transmission occurs between wild animals, with humans forming dead end hosts if infected. It is worth mentioning that sandflies use wild animal burrows as breeding sites (Feliciangeli, 2004). Thus, it has been suggested that in the case of *Phlebotomus perniciosus* a possible overlapping of wild and domestic environments may occur due to the widespread presence of this sandfly species in both environments (Millán et al., 2014). In the strictly anthroponotic transmission cycles humans with kala-azar, post kala-azar dermal leishmaniasis (PKDL) and to a lesser extent those with asymptomatic infection are the sole reservoirs (Bern et al., 2008; Quinnell and Courtenay, 2009). The specific *Leishmania* spp are transmitted either anthroponotically or zoonotically. However, exceptions such as *Leishmania tropica* which although having a predominant anthroponotic cycle, may be transmitted from animals, also exist (Pace, 2014).

In humans, the majority of *Leishmania* infections lead to asymptomatic cases. However, when the disease is declared, it is expressed in four main clinical forms according to the location of the parasite in mammalian tissues—referred to as visceral, cutaneous,

diffuse cutaneous, and mucocutaneous leishmaniasis (Alvar et al., 2012; Bañuls et al., 2007). In dogs infection manifests itself as a chronic subclinical infection, self-limiting disease, or non-self-limiting disease (Solano-Gallego et al., 2009). In endemic areas, most dogs appear to have chronic infections that last for as long as they live (Oliva et al., 2006) and only a small proportion end up developing severe disease (Baneth et al., 2008). Similarly, *Leishmania* infected wild mammals may be symptomatic or asymptomatic. Canids may present cutaneous crusts, ulcers and alopecia. Emaciation, pale mucous membranes, liver enlargement and discoloration, splenomegaly and lymphadenopathy are among the common findings at necropsy. Pulmonary congestion and edema, hemorrhages in skin, lung, heart, and intestines are among the less specific changes reported (Beck et al., 2008; Luppi et al., 2008; Malta et al., 2010; Souza et al., 2010, 2014).

*Leishmania* infection has been documented in various wild mammalian species, including carnivores (Beck et al., 2008; Luppi et al., 2008), primates (Malta et al., 2010), marsupials (Santiago et al., 2007), edentates (De Araújo et al., 2013), lagomorphs (Molina et al., 2012; F. Ruiz-Fons et al., 2013; Tsokana et al., 2015), bats (De Lima et al., 2008) and rodents (Kassahun et al., 2015; Papadogiannakis et al., 2010). However, a fundamental factor determining the ability of a species to act as a potential reservoir is its infectiousness to sandflies. In Europe, this capacity has been demonstrated for the black rat (*Rattus rattus*) in Italy (Gradoni et al., 1983) and the Iberian hare in Spain (Molina et al., 2012).

The first reference of infected hares came from China in Yarkend hare (*Lepus yarkandensis*) (Liao LiFu et al., 2009). At late 2010, an outbreak of human leishmaniasis cases (a surprising 446 human clinical cases were recorded), centred on a suburban park, in Fuenlabrada, southwestern Madrid (Spain), as well as the unchanged incidence in dogs in the same period, prompted the researchers to investigate the role of the Iberian hare in the epidemiology of *L. infantum*. According to different authors, in that municipality, 30% to 45% of hares were *Leishmania* infected (Arce et al., 2013; Moreno et al., 2014). The molecular typing of hare isolates showed that the isolates involved in the outbreak belonged to the ITS LOMBARDI subtype of *L. infantum*, as those isolated in different parts of Madrid since at least 1992 (Chicharro et al., 2013). Xenodiagnosis showed that seven apparently healthy naturally infected hares were infectious to a

mean 4.7% (0–10.6%) *Phlebotomus perniciosus*, a competent vector for *L. infantum* suggesting that hares may represent at least a secondary reservoir in the sylvatic transmission cycle of *L.infantum* in that region (Molina et al., 2012). In another study, analysis of blood meals of ten sand flies captured in the affected area showed a high preference for hares (n=6), followed by humans (n=3), and cats (n=1) (Jiménez et al., 2013). Following studies in Spain showed that *L. granatensis, L. europaeus, L. castroviejoi* from six different regions, presented an overall 43.6% DNA prevalence (*Le. granatensis* 42.1%, *Le. europaeus* 56.3%) (F Ruiz-Fons et al., 2013). Later, *Leishmania* infection in hares was found to be 23.49% in Northern Greece (Tsokana et al., 2015), 18.52% in Italy (Zanet et al., 2016) and more recently 1.9% in central Italy (Rocchigiani et al., 2018). A seroprevalence of 74.1% was reported in *Le. granatensis* in Spain (Moreno et al., 2014) while in Italy the seroprevalence in *Le. europaeus* was 0.9% (Ebani et al., 2016). Clinical illness has not been detected in seropositive hares (Moreno et al., 2014). It has been suggested that possibly, under specific circumstances such as an unusually high concentration of hares, high density of sandflies and a low level of immunity in the human population (Carrillo et al., 2013) this species can act as reservoir for *L. infantum* infection (Millán et al., 2014).

## References


Abrantes, J., Lopes, A.M., Dalton, K.P., Melo, P., Correia, J.J., Ramada, M., Alves, P.C., Parra, F., Esteves, P.J., 2013. New Variant of Rabbit Hemorrhagic Disease Virus, Portugal, 2012–2013. Emerg. Infect. Dis. 19. https://doi.org/10.3201/eid1911.130908

Abravanel, F., Lhomme, S., El Costa, H., Schvartz, B., Peron, J.-M., Kamar, N., Izopet, J., 2017. Rabbit Hepatitis E Virus Infections in Humans, France. Emerg. Infect. Dis. 23, 1191–1193. https://doi.org/10.3201/eid2307.170318

Akhoundi, M., Kuhls, K., Cannet, A., Votýpka, J., Marty, P., Delaunay, P., Sereno, D., 2016. A Historical Overview of the Classification, Evolution, and Dispersion of Leishmania Parasites and Sandflies. PLoS Negl. Trop. Dis. 10, e0004349. https://doi.org/10.1371/journal.pntd.0004349

Alvar, J., Vélez, I.D., Bern, C., Herrero, M., Desjeux, P., Cano, J., Jannin, J., den Boer, M., WHO Leishmaniasis Control Team, 2012. Leishmaniasis worldwide and global estimates of its incidence. PloS One 7, e35671. https://doi.org/10.1371/journal.pone.0035671

Alves, P.C., Ferrand, N., Hackländer, K. (Eds.), 2008. Lagomorph biology: evolution, ecology, and conservation. Springer, Berlin ; New York.

Anagnostou, V., Papa, A., 2009. Evolution of Crimean-Congo Hemorrhagic Fever virus. Infect. Genet. Evol. 9, 948–954. https://doi.org/10.1016/j.meegid.2009.06.018

Anheyer-Behmenburg, H.E., Szabo, K., Schotte, U., Binder, A., Klein, G., Johne, R., 2017. Hepatitis E Virus in Wild Boars and Spillover Infection in Red and Roe Deer, Germany, 2013–2015. Emerg. Infect. Dis. 23, 130–133. https://doi.org/10.3201/eid2301.161169

Aradaib, I.E., Erickson, B.R., Mustafa, M.E., Khristova, M.L., Saeed, N.S., Elageb, R.M., Nichol, S.T., 2010. Nosocomial Outbreak of Crimean-Congo Hemorrhagic Fever, Sudan. Emerg. Infect. Dis. 16, 837–839. https://doi.org/10.3201/eid1605.091815

Arce, A., Estirado, A., Ordobas, M., Sevilla, S., García, N., Moratilla, L., de la Fuente, S., Martínez, A.M., Pérez, A.M., Aránguez, E., Iriso, A., Sevillano, O., Bernal, J., Vilas, F., 2013. Re-emergence of leishmaniasis in Spain: community outbreak in Madrid, Spain, 2009 to 2012. Euro Surveill. Bull. Eur. Sur Mal. Transm. Eur. Commun. Dis. Bull. 18, 20546.

Aubert, D., Ajzenberg, D., Richomme, C., Gilot-Fromont, E., Terrier, M.E., de Gevigney, C., Game, Y., Maillard, D., Gibert, P., Dardé, M.L., Villena, I., 2010. Molecular and biological characteristics of Toxoplasma gondii isolates from wildlife in France. Vet. Parasitol. 171, 346–349. https://doi.org/10.1016/j.vetpar.2010.03.033

Bachofen, C., Grant, D.M., Willoughby, K., Zadoks, R.N., Dagleish, M.P., Russell, G.C., 2014. Experimental infection of rabbits with bovine viral diarrhoea virus by a natural route of exposure. Vet. Res. 45, 34. https://doi.org/10.1186/1297-9716-45-34

Baily, J.L., Dagleish, M.P., Graham, M., Maley, M., Rocchi, M.S., 2014. RHDV variant 2 presence detected in Scotland. Vet. Rec. 174, 411. https://doi.org/10.1136/vr.g2781

Bakaloudis, D.E., Bontzorlos, V.A., Vlachos, C.G., Papakosta, M.A., Chatzinikos, E.N., Braziotis, S.G., Kontsiotis, V.J., 2015. Factors affecting the diet of the red fox (Vulpes vulpes) in a heterogeneousMediterranean landscape. Turk. J. Zool. 39, 1151–1159. https://doi.org/10.3906/zoo-1411-22

Bakaloudis, D.E., Vlachos, C.G., Papakosta, M.A., Bontzorlos, V.A., Chatzinikos, E.N., 2012. Diet Composition and Feeding Strategies of the Stone Marten ( *Martes foina* ) in a Typical Mediterranean Ecosystem. Sci. World J. 2012, 1–11. https://doi.org/10.1100/2012/163920

Baneth, G., Koutinas, A.F., Solano-Gallego, L., Bourdeau, P., Ferrer, L., 2008. Canine leishmaniosis - new concepts and insights on an expanding zoonosis: part one. Trends Parasitol. 24, 324–330. https://doi.org/10.1016/j.pt.2008.04.001

Bañuls, A.-L., Hide, M., Prugnolle, F., 2007. Leishmania and the leishmaniases: a parasite genetic update and advances in taxonomy, epidemiology and pathogenicity in humans. Adv. Parasitol. 64, 1–109. https://doi.org/10.1016/S0065-308X(06)64001-3

Barlow, A., Lawrence, K., Everest, D., Dastjerdi, A., Finnegan, C., Steinbach, F., 2014. Confirmation of myxomatosis in a European brown hare in Great Britain: Myxomatosis. Vet. Rec. 175, 75–76. https://doi.org/10.1136/vr.g4621

Bartling, C., Wölfel, R., Nikolaou, K., Petry, T., Thiede, S., Hildebrandt, T., Fassbender, M., Göritz, F., Blottner, S., Spittler, H., Neubauer, H., 2004. [Prevalence of anti-Yersinia antibodies in European brown hares in North-Rhine Westphalia, Germany]. DTW Dtsch. Tierarztl. Wochenschr. 111, 259–260, 262–264.

Bartova, E., Sedlak, K., 2012. Toxoplasmosis in Animals in the Czech Republic - The Last 10 Years, in: Djurkovi Djakovi, O. (Ed.), Toxoplasmosis - Recent Advances. InTech.

Bártová, E., Sedlák, K., Treml, F., Holko, I., Literák, I., 2010. Neospora caninum and Toxoplasma gondii antibodies in European brown hares in the Czech Republic, Slovakia and Austria. Vet. Parasitol. 171, 155–158. https://doi.org/10.1016/j.vetpar.2010.03.002

Becher, P., Orlich, M., Shannon, A.D., Horner, G., König, M., Thiel, H.J., 1997. Phylogenetic analysis of pestiviruses from domestic and wild ruminants. J. Gen. Virol. 78 ( Pt 6), 1357–1366. https://doi.org/10.1099/0022-1317-78-6-1357

Beck, A., Beck, R., Kusak, J., Gudan, A., Martinkovic, F., Artukovic, B., Hohsteter, M., Huber, D., Marinculic, A., Grabarevic, Z., 2008. A case of visceral leishmaniosis in a gray wolf (Canis lupus) from Croatia. J. Wildl. Dis. 44, 451–456. https://doi.org/10.7589/0090-3558-44.2.451

Bengis, R.G., Kock, R.A., Fischer, J., 2002. Infectious animal diseases: the wildlife/livestock interface. Rev. Sci. Tech. Int. Off. Epizoot. 21, 53–65.

Bern, C., Maguire, J.H., Alvar, J., 2008. Complexities of Assessing the Disease Burden Attributable to Leishmaniasis. PLoS Negl. Trop. Dis. 2, e313. https://doi.org/10.1371/journal.pntd.0000313

Billinis, C., Psychas, V., Tontis, D.K., Spyrou, V., Birtsas, P.K., Sofia, M., Likotrafitis, F., Maslarinou, O.M., Kanteres, D., 2005. European brown hare syndrome in wild European brown hares from Greece. J. Wildl. Dis. 41, 783–786. https://doi.org/10.7589/0090-3558-41.4.783

Bogdanovich, T., Carniel, E., Fukushima, H., Skurnik, M., 2003. Use of O-antigen gene cluster-specific PCRs for the identification and O-genotyping of Yersinia pseudotuberculosis and Yersinia pestis. J. Clin. Microbiol. 41, 5103–5112.

Bonardi, S., Bruini, I., D'Incau, M., Van Damme, I., Carniel, E., Brémont, S., Cavallini, P., Tagliabue, S., Brindani, F., 2016. Detection, seroprevalence and antimicrobial resistance of Yersinia enterocolitica and Yersinia pseudotuberculosis in pig tonsils in Northern Italy. Int. J. Food Microbiol. 235, 125–132. https://doi.org/10.1016/j.ijfoodmicro.2016.07.033

Bray, Y., Devillard, S., Marboutin, E., Mauvy, B., Péroux, R., 2007. Natal dispersal of European hare in France. J. Zool. 273, 426–434. https://doi.org/10.1111/j.1469-7998.2007.00348.x

Broekhuizen, S., Maaskamp, F., 1980. Behaviour of does and leverets of the European hare (Lepus europaem) whilst nursing. J. Zool. 191, 487–501. https://doi.org/10.1111/j.1469-7998.1980.tb01480.x

Camarda, A., Pugliese, N., Cavadini, P., Circella, E., Capucci, L., Caroli, A., Legretto, M., Mallia, E., Lavazza, A., 2014. Detection of the new emerging rabbit haemorrhagic disease type 2 virus (RHDV2) in Sicily from rabbit (Oryctolagus cuniculus) and Italian hare (Lepus corsicanus). Res. Vet. Sci. 97, 642–645. https://doi.org/10.1016/j.rvsc.2014.10.008

Carrillo, E., Moreno, J., Cruz, I., 2013. What is responsible for a large and unusual outbreak of leishmaniasis in Madrid? Trends Parasitol. 29, 579–580. https://doi.org/10.1016/j.pt.2013.10.007

Caruso, C., Peletto, S., Rosamilia, A., Modesto, P., Chiavacci, L., Sona, B., Balsamelli, F., Ghisetti, V., Acutis, P.L., Pezzoni, G., Brocchi, E., Vitale, N., Masoero, L., 2017. Hepatitis E Virus: A Cross-Sectional Serological and Virological Study in Pigs and Humans at Zoonotic Risk within a High-Density Pig Farming Area. Transbound. Emerg. Dis. 64, 1443–1453. https://doi.org/10.1111/tbed.12533

Catar, G., 1972. Studies on Toxoplasmosis as regards its natural focality in Slovakia. Folia Parasitol. (Praha) 253–256.

Chae, C., Choi, C., 2002. Localization of classical swine fever virus in male gonads during subclinical infection. J. Gen. Virol. 83, 2717–2721. https://doi.org/10.1099/0022-1317-83-11-2717

Chapman, J.A., Flux, J.E.C., International Union for Conservation of Nature and Natural Resources (Eds.), 1990. Rabbits, hares, and pikas: status survey and conservation action plan. International Union for Conservation of Nature and Natural Resources, Gland, Switzerland.

Chasey, D., Lucas, M., Westcott, D., Williams, M., 1992. European brown hare syndrome in the U.K.; a calicivirus related to but distinct from that of viral haemorrhagic disease in rabbits. Arch. Virol. 124, 363–370. https://doi.org/10.1007/BF01309816

Chaudhuri, R.R., Ren, C.-P., Desmond, L., A. Vincent, G., Silman, N.J., Brehm, J.K., Elmore, M.J., Hudson, M.J., Forsman, M., Isherwood, K.E., Guryčová, D., Minton, N.P., Titball, R.W., Pallen, M.J., Vipond, R., 2007. Genome Sequencing Shows that European Isolates of Francisella tularensis Subspecies tularensis Are Almost Identical to US Laboratory Strain Schu S4. PLoS ONE 2, e352. https://doi.org/10.1371/journal.pone.0000352

Chiari, M., Ferrari, N., Giardiello, D., Avisani, D., Zanoni, M., Alborali, G.L., Lanfranchi, P., Guberti, V., Lorenzo, C., Antonio, L., 2014. Temporal dynamics of European brown hare syndrome infection in Northern Italian brown hares (Lepus europaeus). Eur. J. Wildl. Res. 60, 891–896. https://doi.org/10.1007/s10344-014-0856-6

Chiari, M., Molinari, S., Cavadini, P., Bertasi, B., Zanoni, M., Capucci, L., Lavazza, A., 2016. Red foxes (Vulpes vulpes) feeding brown hares (Lepus europaeus) infected by European brown hare syndrome virus (EBHSv) might be involved in the spread of the virus. Eur. J. Wildl. Res. 62, 761–765. https://doi.org/10.1007/s10344-016-1055-4

Chicharro, C., Llanes-Acevedo, I.P., García, E., Nieto, J., Moreno, J., Cruz, I., 2013. Molecular typing of Leishmania infantum isolates from a leishmaniasis outbreak in Madrid, Spain, 2009 to 2012. Euro Surveill. Bull. Eur. Sur Mal. Transm. Eur. Commun. Dis. Bull. 18, 20545.

Chiesa, C., Pacifico, L., Nanni, F., Renzi, A.M., Ravagnan, G., 1993. Yersinia pseudotuberculosis in Italy. Attempted recovery from 37,666 samples. Microbiol. Immunol. 37, 391–394.

Chomel, B.B., Belotto, A., Meslin, F.-X., 2007. Wildlife, Exotic Pets, and Emerging Zoonoses1. Emerg. Infect. Dis. 13, 6–11. https://doi.org/10.3201/eid1301.060480

Christiansen, M., Siim, J.C., 1951. Toxoplasmosis in hares in Denmark. The Lancet 257, 1201–1203. https://doi.org/10.1016/S0140-6736(51)92707-9

Collins, J.J., 1955. Myomatosis in the common hare. Irish Veterinary Journal 9, 268.

Colom-Cadena, A., Cabezón, O., Rosell, R., Fernández-Aguilar, X., Blanch-Lázaro, B., Tetas, E., Lavín, S., Marco, I., 2016. The European hare ( Lepus europaeus ) as a potential wild reservoir for ruminant pestiviruses. Prev. Vet. Med. 131, 60–63. https://doi.org/10.1016/j.prevetmed.2016.06.014

Cook, W.E., Williams, E.S., Thorne, E.T., Kreeger, T.J., Stout, G., Bardsley, K., Edwards, H., Schurig, G., Colby, L.A., Enright, F., Elzer, P.H., 2002. Brucella abortus strain RB51 vaccination in elk. I. Efficacy of reduced dosage. J. Wildl. Dis. 38, 18–26. https://doi.org/10.7589/0090-3558-38.1.18

Dalton, K.P., Nicieza, I., Balseiro, A., Muguerza, M.A., Rosell, J.M., Casais, R., Álvarez, Á.L., Parra, F., 2012. Variant Rabbit Hemorrhagic Disease Virus in Young Rabbits, Spain. Emerg. Infect. Dis. 18, 2009–2012. https://doi.org/10.3201/eid1812.120341

Dantas-Torres, F., Lorusso, V., Testini, G., de Paiva-Cavalcanti, M., Figueredo, L.A., Stanneck, D., Mencke, N., Brandão-Filho, S.P., Alves, L.C., Otranto, D., 2010. Detection of Leishmania infantum in Rhipicephalus sanguineus ticks from Brazil and Italy. Parasitol. Res. 106, 857–860. https://doi.org/10.1007/s00436-010-1722-4

De Araújo, V.A.L., Boité, M.C., Cupolillo, E., Jansen, A.M., Roque, A.L.R., 2013. Mixed infection in the anteater Tamandua tetradactyla (Mammalia: Pilosa) from Pará State, Brazil: Trypanosoma cruzi, T. rangeli and Leishmania infantum. Parasitology 140, 455–460. https://doi.org/10.1017/S0031182012001886

De Lima, H., Rodríguez, N., Barrios, M.A., Avila, A., Cañizales, I., Gutiérrez, S., 2008. Isolation and molecular identification of Leishmania chagasi from a bat (Carollia perspicillata) in northeastern Venezuela. Mem. Inst. Oswaldo Cruz 103, 412–414.

Di Profio, F., Melegari, I., Sarchese, V., Robetto, S., Bermudez Sanchez, S., Carella, E., Orusa, R., Cavadini, P., Lavazza, A., Marsilio, F., Martella, V., Di Martino, B., 2018. Potential role of wolf (Canis lupus) as passive carrier of European brown hare syndrome virus (EBHSV). Res. Vet. Sci. 117, 81–84. https://doi.org/10.1016/j.rvsc.2017.11.016

Dobler, G., Wölfel, R., Schmüser, H., Essbauer, S., Pfeffer, M., 2006. Seroprevalence of tick-borne and mosquito-borne arboviruses in European brown hares in Northern and Western Germany. Int. J. Med. Microbiol. 296, 80–83. https://doi.org/10.1016/j.ijmm.2005.12.007

Dremsek, P., Wenzel, J.J., Johne, R., Ziller, M., Hofmann, J., Groschup, M.H., Werdermann, S., Mohn, U., Dorn, S., Motz, M., Mertens, M., Jilg, W., Ulrich, R.G., 2012. Seroprevalence study in forestry workers from eastern Germany using novel genotype 3- and rat hepatitis E virus-specific immunoglobulin G ELISAs. Med. Microbiol. Immunol. (Berl.) 201, 189–200. https://doi.org/10.1007/s00430-011-0221-2

Drews, B., Szentiks, C.A., Roellig, K., Fickel, J., Schroeder, K., Duff, J.P., Lavazza, A., Hildebrandt, T.B., Goeritz, F., 2011. Epidemiology, control and management of an EBHS outbreak in captive hares. Vet. Microbiol. 154, 37–48. https://doi.org/10.1016/j.vetmic.2011.06.021

Dubey, J.P., Beattie, C.P., 1988. Toxoplasmosis of animals and man. CRC Press, Boca Raton, Fla.

Dubey, J.P., Dubey, J.P., 2010. Toxoplasmosis of animals and humans, 2nd ed. ed. CRC Press, Boca Raton.

Dubey, J.P., Jones, J.L., 2008. Toxoplasma gondii infection in humans and animals in the United States. Int. J. Parasitol. 38, 1257–1278. https://doi.org/10.1016/j.ijpara.2008.03.007

Duff, J.P., Chasey, D., Munro, R., Wooldridge, M., 1994. European brown hare syndrome in England. Vet. Rec. 134, 669–673.

Ebani, V.V., Poli, A., Rocchigiani, G., Bertelloni, F., Nardoni, S., Papini, R.A., Mancianti, F., 2016. Serological survey on some pathogens in wild brown hares (Lepus europaeus) in Central Italy. Asian Pac. J. Trop. Med. 9, 465–469. https://doi.org/10.1016/j.apjtm.2016.03.032

Edelhofer, R., Heppe-Winger, E.M., Haßl, A., Aspöck, H., 1989. Toxoplasma infections in game in Eastern Austria. Mitt. Österr. Ges. Tropenmed. Parasitol. 119–123.

Eskens, U., Frölich, K., Kugel, B., Frost, J.W., Streich, W.J., Bensinger, S., 2000. Seroepidemiologische Untersuchungen zur Verbreitung des European Brown Hare Syndrome (EBHS) und der Rabbit Haemorrhagic Disease (RHD) in Feldhasenbeständen ausgewählter Reviere in der Bundesrepublik Deutschland. Z. Für Jagdwiss. 46, 61–72. https://doi.org/10.1007/BF02241569

Estrada-Peña, A., Jameson, L., Medlock, J., Vatansever, Z., Tishkova, F., 2012a. Unraveling the ecological complexities of tick-associated Crimean-Congo hemorrhagic fever virus transmission: a gap analysis for the western Palearctic. Vector Borne Zoonotic Dis. Larchmt. N 12, 743–752. https://doi.org/10.1089/vbz.2011.0767

Estrada-Peña, A., Sánchez, N., Estrada-Sánchez, A., 2012b. An assessment of the distribution and spread of the tick Hyalomma marginatum in the western

Palearctic under different climate scenarios. Vector Borne Zoonotic Dis. Larchmt. N 12, 758–768. https://doi.org/10.1089/vbz.2011.0771

Etheredge, G.D., Michael, G., Muehlenbein, M.P., Frenkel, J.K., 2004. The roles of cats and dogs in the transmission of Toxoplasma infection in Kuna and Embera children in eastern Panama. Rev. Panam. Salud Publica Pan Am. J. Public Health 16, 176–186.

European Food Safety Authority (EFSA), 2009. Porcine brucellosis (Brucella suis): Porcine brucellosis (Brucella suis). EFSA J. 7, 1144. https://doi.org/10.2903/j.efsa.2009.1144

Fagbami, A.H., Tomori, O., Fabiyi, A., Isoun, T.T., 1975. Experimantal Congo virus (Ib - AN 7620) infection in primates. Virologie 26, 33–37.

Feliciangeli, M.D., 2004. Natural breeding places of phlebotomine sandflies. Med. Vet. Entomol. 18, 71–80.

Fenner, F., 1965. VIRUSES OF THE MYXOMA-FIBROMA SUBGROUP OF THE POXVIRUSES. II. COMPARISON OF SOLUBLE ANTIGENS BY GEL DIFFUSION TESTS, AND A GENERAL DISCUSSION OF THE SUBGROUP. Aust. J. Exp. Biol. Med. Sci. 43, 143–156.

Fenner, F., Fantini, B., 1999. Biological Control of Vertebrate Pests. The History of Myxomatosis – an Experiment in Evolution. Med Hist 45, 139–140.

Fernández-Aguilar, X., Alzaga, V., Villanúa, D., Cabezón, O., García-Bocanegra, I., Dubey, J.P., Almería, S., 2013. Epidemiology and prevalence of Toxoplasma gondii infection in the Iberian hare (Lepus granatensis). Vet. Parasitol. 196, 194–198. https://doi.org/10.1016/j.vetpar.2013.01.061

Fort, M., Baldone, V., Fuchs, L., Giménez, H., Rojas, M., Breccia, J.D., Oyhenart, J., 2012. Experimental infection of rabbits (Oryctolagus cuniculus) with Brucella suis biovar 1 isolated from wild hares (Lepus europaeus). Vet. Microbiol. 156, 439–442. https://doi.org/10.1016/j.vetmic.2011.11.006

Foster, G., Osterman, B.S., Godfroid, J., Jacques, I., Cloeckaert, A., 2007. Brucella ceti sp. nov. and Brucella pinnipedialis sp. nov. for Brucella strains with cetaceans and seals as their preferred hosts. Int. J. Syst. Evol. Microbiol. 57, 2688–2693. https://doi.org/10.1099/ijs.0.65269-0

Frandölich, K., Wisser, J., Schmüser, H., Fehlberg, U., Neubauer, H., Grunow, R., Nikolaou, K., Priemer, J., Thiede, S., Streich, W.J., Speck, S., 2003. EPIZOOTIOLOGIC AND ECOLOGIC INVESTIGATIONS OF EUROPEAN BROWN HARES (LEPUS EUROPAEUS) IN SELECTED POPULATIONS FROM SCHLESWIG-HOLSTEIN, GERMANY. J. Wildl. Dis. 39, 751–761. https://doi.org/10.7589/0090-3558-39.4.751

Fratini, F., Verin, R., Ebani, V.V., Ambrogi, C., Bertelloni, F., Turchi, B., Poli, A., Cerri, D., 2017. Experimental infection with Yersinia pseudotuberculosis in European brown hare ( Lepus europaeus , Pallas). Asian Pac. J. Trop. Med. 10, 285–291. https://doi.org/10.1016/j.apjtm.2017.03.008

Frölich, K., Fickel, J., Ludwig, A., Lieckfeldt, D., Streich, W.J., Jurcik, R., Slamecka, J., Wibbelt, G., 2007. New variants of European brown hare syndrome virus strains in free-ranging European brown hares (Lepus europaeus) from Slovakia. J. Wildl. Dis. 43, 89–96. https://doi.org/10.7589/0090-3558-43.1.89

Frölich, K., Haerer, G., Bacciarini, L., Janovsky, M., Rudolph, M., Giacometti, M., 2001. European brown hare syndrome in free-ranging European brown and mountain hares from Switzerland. J. Wildl. Dis. 37, 803–807. https://doi.org/10.7589/0090-3558-37.4.803

Frölich, K., Jürgen Streich, W., 1998. Serologic Evidence of Bovine Viral Diarrhea Virus in Free-ranging Rabbits from Germany. J. Wildl. Dis. 34, 173–178. https://doi.org/10.7589/0090-3558-34.1.173

Frölich, K., Kujawski, O.E.J.G., Rudolph, M., Ronsholt, L., Speck, S., 2003a. European brown hare syndrome virus in free-ranging European brown hares from Argentina. J. Wildl. Dis. 39, 121–124. https://doi.org/10.7589/0090-3558-39.1.121

Frölich, K., Meyer, H.H., Pielowski, Z., Ronsholt, L., von Seck-Lanzendorf, S., Stolte, M., 1996. European brown hare syndrome in free-ranging hares in Poland. J. Wildl. Dis. 32, 280–285. https://doi.org/10.7589/0090-3558-32.2.280

Frölich, K., Wisser, J., Schmüser, H., Fehlberg, U., Neubauer, H., Grunow, R., Nikolaou, K., Priemer, J., Thiede, S., Streich, W.J., Speck, S., 2003b. Epizootiologic and ecologic investigations of European brown hares (Lepus europaeus) in

selected populations from Schleswig-Holstein, Germany. J. Wildl. Dis. 39, 751–761. https://doi.org/10.7589/0090-3558-39.4.751

Fukushima, H., 1992. Direct isolation of Yersinia pseudotuberculosis from fresh water in Japan. Appl. Environ. Microbiol. 58, 2688–2690.

Fukushima, H., Gomyoda, M., Tsubokura, M., Aleksić, S., 1995. Isolation of Yersinia pseudotuberculosis from river waters in Japan and Germany using direct KOH and HeLa cell treatments. Zentralblatt Für Bakteriol. 282, 40–49. https://doi.org/10.1016/S0934-8840(11)80795-4

Fukushima, H., Matsuda, Y., Seki, R., Tsubokura, M., Takeda, N., Shubin, F.N., Paik, I.K., Zheng, X.B., 2001. Geographical heterogeneity between Far Eastern and Western countries in prevalence of the virulence plasmid, the superantigen Yersinia pseudotuberculosis-derived mitogen, and the high-pathogenicity island among Yersinia pseudotuberculosis strains. J. Clin. Microbiol. 39, 3541–3547. https://doi.org/10.1128/JCM.39.10.3541-3547.2001

Fukushima, H., Shimizu, S., Inatsu, Y., 2011. *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* Detection in Foods. J. Pathog. 2011, 1–9. https://doi.org/10.4061/2011/735308

Gamarra, J.A., Cabezón, O., Pabón, M., Arnal, M.C., Luco, D.F., Dubey, J.P., Gortázar, C., Almeria, S., 2008. Prevalence of antibodies against Toxoplasma gondii in roe deer from Spain. Vet. Parasitol. 153, 152–156. https://doi.org/10.1016/j.vetpar.2008.01.028

Gavier-Widén, D., 1994. Morphologic and immunohistochemical characterization of the hepatic lesions associated with European brown hare syndrome. Vet. Pathol. 31, 327–334. https://doi.org/10.1177/030098589403100305

Gavier-Widén, D., Mörner, T., 1991. Epidemiology and diagnosis of the European brown hare syndrome in Scandinavian countries: a review. Rev. Sci. Tech. Int. Off. Epizoot. 10, 453–458.

Godfroid, J., 2012. Brucellosis, in: Gavier-Widén, D., Duff, J.P., Meredith, A. (Eds.), Infectious Diseases of Wild Mammals and Birds in Europe. Wiley-Blackwell, Oxford, UK, pp. 318–328. https://doi.org/10.1002/9781118342442.ch24

Godfroid, J., Cloeckaert, A., Liautard, J.-P., Kohler, S., Fretin, D., Walravens, K., Garin-Bastuji, B., Letesson, J.-J., 2005. From the discovery of the Malta fever?s agent

to the discovery of a marine mammal reservoir, brucellosis has continuously been a re-emerging zoonosis. Vet. Res. 36, 313–326. https://doi.org/10.1051/vetres:2005003

Godfroid, J., Garin-Bastuji, B., Saegerman, C., Blasco, J.M., 2013. Brucellosis in terrestrial wildlife. Rev. Sci. Tech. Int. Off. Epizoot. 32, 27–42.

Gradoni, L., Pozio, E., Gramiccia, M., Maroli, M., Bettini, S., 1983. Leishmaniasis in Tuscany (Italy): VII. Studies on the role of the black rat, Rattus rattus, in the epidemiology of visceral leishmaniasis. Trans. R. Soc. Trop. Med. Hyg. 77, 427–431.

Grant, D.M., Dagleish, M.P., Bachofen, C., Boag, B., Deane, D., Percival, A., Zadoks, R.N., Russell, G.C., 2015. Assessment of the rabbit as a wildlife reservoir of bovine viral diarrhea virus: serological analysis and generation of trans-placentally infected offspring. Front. Microbiol. 6. https://doi.org/10.3389/fmicb.2015.01000

Grilli, G., Piccirillo, A., Pisoni, A.M., Cerioli, M., Gallazzi, D., Lavazza, A., 2003. Re-emergence of fibromatosis in farmed game hares (Lepus europaeus) in Italy. Vet. Rec. 153, 152–153.

Gustafsson, K., 1997. Studies of toxoplasmosis in hares and capercaillie. Acta Universitatis Agriculturae Sueciae. Veterinaria, Sweden.

Gustafsson, K., Uggla, A., 1994. Serologic survey for Toxoplasma gondii infection in the Brown hare (Lepus Europaeus P.) in Sweden. J. Wildl. Dis. 30, 201–204. https://doi.org/10.7589/0090-3558-30.2.201

Gustafsson, K., Uggla, A., Järplid, B., 1997. Toxoplasma gondii infection in the mountain hare (Lepus timidus) and domestic rabbit (Oryctolagus cuniculus). I. Pathology. J. Comp. Pathol. 117, 351–360.

Gustafsson, K., Uggla, A., Svensson, T., Sjöland, L., 1988. Detection of Toxoplasma gondii in liver tissue sections from brown hares (Lepus europaeus P.) and mountain hares (Lepus timidus L.) using the peroxidase anti-peroxidase (PAP) technique as a complement to conventional histopathology. Zentralblatt Vet. Reihe B J. Vet. Med. Ser. B 35, 402–407.

Gyuranecz, M., 2012. Tularaemia, in: Gavier-Widén, D., Duff, J.P., Meredith, A. (Eds.), Infectious Diseases of Wild Mammals and Birds in Europe. Wiley-Blackwell, Oxford, UK, pp. 303–309. https://doi.org/10.1002/9781118342442.ch22

Gyuranecz, M., Erdélyi, K., Makrai, L., Fodor, L., Szépe, B., Mészáros, Á.R., Dán, A., Dencső, L., Fassang, E., Szeredi, L., 2011. Brucellosis of the European Brown Hare (Lepus europaeus). J. Comp. Pathol. 145, 1–5. https://doi.org/10.1016/j.jcpa.2010.11.013

Gyuranecz, M., Reiczigel, J., Krisztalovics, K., Monse, L., Szabóné, G.K., Szilágyi, A., Szépe, B., Makrai, L., Magyar, T., Bhide, M., Erdélyi, K., 2012. Factors Influencing Emergence of Tularemia, Hungary, 1984–2010. Emerg. Infect. Dis. 18, 1379–1381. https://doi.org/10.3201/eid1808.111826

Gyuranecz, Mikl?s, Rig?, K., D?n, ?d?m, F?ldv?ri, G., Makrai, L., D?nes, B., Fodor, L., Majoros, G., Tirj?k, L., Erd?lyi, K., 2011. Investigation of the Ecology of *Francisella tularensis* During an Inter-Epizootic Period. Vector-Borne Zoonotic Dis. 11, 1031–1035. https://doi.org/10.1089/vbz.2010.0091

Gyuranecz, M., Szeredi, L., Makrai, L., Fodor, L., Mészáros, á. R., Szépe, B., Füleki, M., Erdélyi, K., 2010. Tularemia of European Brown Hare ( *Lepus europaeus* ): A Pathological, Histopathological, and Immunohistochemical Study. Vet. Pathol. 47, 958–963. https://doi.org/10.1177/0300985810369902

Haghdoost, A., Chinikar, S., Khakifirouz, S., Mostafavi, E., 2013. Spatial Analysis of Crimean Congo Hemorrhagic Fever in Iran. Am. J. Trop. Med. Hyg. 89, 1135–1141. https://doi.org/10.4269/ajtmh.12-0509

Hall, R.N., Peacock, D.E., Kovaliski, J., Mahar, J.E., Mourant, R., Piper, M., Strive, T., 2017. Detection of RHDV2 in European brown hares ( *Lepus europaeus* ) in Australia. Vet. Rec. 180, 121.2-121. https://doi.org/10.1136/vr.104034

Hammerschmidt, F., Schwaiger, K., Dähnert, L., Vina-Rodriguez, A., Höper, D., Gareis, M., Groschup, M.H., Eiden, M., 2017. Hepatitis E virus in wild rabbits and European brown hares in Germany. Zoonoses Public Health. https://doi.org/10.1111/zph.12355

Hannam, D.A., 1993. Bovine abortion associated with Yersinia pseudotuberculosis. Vet. Rec. 133, 372.

Hartemink, N.A., Randolph, S.E., Davis, S.A., Heesterbeek, J.A.P., 2008. The Basic Reproduction Number for Complex Disease Systems: Defining $R_0$ for Tick-Borne Infections. Am. Nat. 171, 743–754. https://doi.org/10.1086/587530

Hoogstraal, H., 1979. The epidemiology of tick-borne Crimean-Congo hemorrhagic fever in Asia, Europe, and Africa. J. Med. Entomol. 15, 307–417.

Hubbert, W.T., 1972. Yersiniosis in mammals and birds in the United States: case reports and review. Am. J. Trop. Med. Hyg. 21, 458–463.

Humolli, I., Dedushaj, I., Zupanac, T.A., Muçaj, S., 2010. Epidemiological, serological and herd immunity of Crimean-Congo haemorrhagic fever in Kosovo. Med. Arh. 64, 91–93.

International Committee on Taxonomy of Viruses, King, A.M.Q. (Eds.), 2012. Virus taxonomy: classification and nomenclature of viruses: ninth report of the International Committee on Taxonomy of Viruses. Academic Press, London ; Waltham, MA.

IUCN, 2008. Lepus europaeus: Smith, A.T. & Johnston, C.H.: The IUCN Red List of Threatened Species 2008: e.T41280A10430693. https://doi.org/10.2305/IUCN.UK.2008.RLTS.T41280A10430693.en

Jackson, J., McGregor, A., Cooley, L., Ng, J., Brown, M., Ong, C.W., Darcy, C., Sintchenko, V., 2012. Francisella tularensis subspecies holarctica, Tasmania, Australia, 2011. Emerg. Infect. Dis. 18, 1484–1486. https://doi.org/10.3201/eid1809.111856

Jacotot, H., Vallee, A., Virat, B., 1954. Sur un cas de myxomatose chez le lievre. Annales de l'Institut Pasteur 86, 105–107.

Jiménez, M., González, E., Iriso, A., Marco, E., Alegret, A., Fúster, F., Molina, R., 2013. Detection of Leishmania infantum and identification of blood meals in Phlebotomus perniciosus from a focus of human leishmaniasis in Madrid, Spain. Parasitol. Res. 112, 2453–2459. https://doi.org/10.1007/s00436-013-3406-3

Johne, R., Dremsek, P., Reetz, J., Heckel, G., Hess, M., Ulrich, R.G., 2014. Hepeviridae: An expanding family of vertebrate viruses. Infect. Genet. Evol. 27, 212–229. https://doi.org/10.1016/j.meegid.2014.06.024

Jokelainen, P., 2012. Endemic Toxoplasma gondii Genotype II Causes Fatal Infections in Animal Hosts in Europe - Lessons Learnt, in: Djurkovi Djakovi, O. (Ed.), Toxoplasmosis - Recent Advances. InTech. https://doi.org/10.5772/49984

Jokelainen, P., Isomursu, M., Näreaho, A., Oksanen, A., 2011. Natural toxoplasma gondii infections in European brown hares and mountain hares in Finland: proportional mortality rate, antibody prevalence, and genetic characterization. J. Wildl. Dis. 47, 154–163. https://doi.org/10.7589/0090-3558-47.1.154

Kamar, N., Rostaing, L., Izopet, J., 2013. Hepatitis E virus infection in immunosuppressed patients: natural history and therapy. Semin. Liver Dis. 33, 62–70. https://doi.org/10.1055/s-0033-1338115

Kamar, N., Selves, J., Mansuy, J.-M., Ouezzani, L., Péron, J.-M., Guitard, J., Cointault, O., Esposito, L., Abravanel, F., Danjoux, M., Durand, D., Vinel, J.-P., Izopet, J., Rostaing, L., 2008. Hepatitis E virus and chronic hepatitis in organ-transplant recipients. N. Engl. J. Med. 358, 811–817. https://doi.org/10.1056/NEJMoa0706992

Karstad, L., Thorsen, J., Davies, G., Kaminjolo, J.S., 1977. Poxvirus fibromas on African hares. J. Wildl. Dis. 13, 245–247.

Kasapidis, P., Suchentrunk, F., Magoulas, A., Kotoulas, G., 2005. The shaping of mitochondrial DNA phylogeographic patterns of the brown hare (Lepus europaeus) under the combined influence of Late Pleistocene climatic fluctuations and anthropogenic translocations. Mol. Phylogenet. Evol. 34, 55–66. https://doi.org/10.1016/j.ympev.2004.09.007

Kassahun, A., Sadlova, J., Dvorak, V., Kostalova, T., Rohousova, I., Frynta, D., Aghova, T., Yasur-Landau, D., Lemma, W., Hailu, A., Baneth, G., Warburg, A., Volf, P., Votypka, J., 2015. Detection of Leishmania donovani and L. tropica in Ethiopian wild rodents. Acta Trop. 145, 39–44. https://doi.org/10.1016/j.actatropica.2015.02.006

Keim, P., Johansson, A., Wagner, D.M., 2007. Molecular epidemiology, evolution, and ecology of Francisella. Ann. N. Y. Acad. Sci. 1105, 30–66. https://doi.org/10.1196/annals.1409.011

King, A.M., Lefkowitz, E., Adams, M.J., Carstens, E.B., 2011. Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier Science, Burlington.

Kreizinger, Z., Foster, J.T., Rónai, Z., Sulyok, K.M., Wehmann, E., Jánosi, S., Gyuranecz, M., 2014. Genetic relatedness of Brucella suis biovar 2 isolates from hares, wild boars and domestic pigs. Vet. Microbiol. 172, 492–498. https://doi.org/10.1016/j.vetmic.2014.05.031

Kunst, P.J.G., van der Wal, R., van Wieren, S., 2001. Home ranges of brown hares in a natural salt marsh: comparisons with agricultural systems. Acta Theriol. (Warsz.) 46, 287–294. https://doi.org/10.1007/BF03192435

Lafenetre, H., Cortez, A., Rioux, J., Pages, A., Vollhardt, Y., Quatrefages, H., 1960. Enzootie de tumeurs cutanees chez le lievre. Bulletin De Lacademie Veterinaire De France 379–389.

Lavazza, A., Cavadini, P., Barbieri, I., Tizzani, P., Pinheiro, A., Abrantes, J., Esteves, P.J., Grilli, G., Gioia, E., Zanoni, M., Meneguz, P., Guitton, J.-S., Marchandeau, S., Chiari, M., Capucci, L., 2015. Field and experimental data indicate that the eastern cottontail (Sylvilagus floridanus) is susceptible to infection with European brown hare syndrome (EBHS) virus and not with rabbit haemorrhagic disease (RHD) virus. Vet. Res. 46, 13. https://doi.org/10.1186/s13567-015-0149-4

Lavazza, A., Scicluna, M.T., Capucci, L., 1996. Susceptibility of hares and rabbits to the European brown hare syndrome virus (EBHSV) and rabbit haemorrhagic disease virus (RHDV) under experimental conditions. Zentralblatt Vet. Reihe B J. Vet. Med. Ser. B 43, 401–410.

Le Gall-Reculé, G., Lavazza, A., Marchandeau, S., Bertagnoli, S., Zwingelstein, F., Cavadini, P., Martinelli, N., Lombardi, G., Guérin, J.-L., Lemaitre, E., Decors, A., Boucher, S., Le Normand, B., Capucci, L., 2013. Emergence of a new lagovirus related to rabbit haemorrhagic disease virus. Vet. Res. 44, 81. https://doi.org/10.1186/1297-9716-44-81

Le Gall-Reculé, G., Zwingelstein, F., Boucher, S., Le Normand, B., Plassiart, G., Portejoie, Y., Decors, A., Bertagnoli, S., Guérin, J.-L., Marchandeau, S., 2011.

Detection of a new variant of rabbit haemorrhagic disease virus in France. Vet. Rec. 168, 137–138. https://doi.org/10.1136/vr.d697

Le Gall-Reculé, G., Zwingelstein, F., Laurent, S., Portejoie, Y., Rasschaert, D., 2006. Molecular epidemiology of European brown hare syndrome virus in France between 1989 and 2003. Arch. Virol. 151, 1713–1721. https://doi.org/10.1007/s00705-006-0754-7

Leinati, L., Mandelli, G., Carrara, O., 1959. Lesioni cutanee nodulari nelle lepri della pianura padana. Atti della Societa Italiana delle Scienze Veterinarie 429–436.

Leinati, L., Mandelli, G., Carrara, O., Cilli, V., Castrucci, G., Scatozza, E., 1961. Ricerche anatomo-istopatologische e virologiche sulla malattia cutanea nodulare delle lepri padane. Bollettino dell'Istituto Sieroterapico Milanese 295–328.

Liao LiFu, Yan ShunSheng, Wuso, B, Wu Min, Xu Bing, Zhang Yong, Hou YanYan, Lei Gang, 2009. Leishmania infantum firstly isolated from Yarkend hare (Lepus yarkandensis). Chinese Journal of Vector Biology and Control 20, 45–47.

Lindenbach, B.D., Murray, C.L., Thiel, H.J., Rice, C.M., 2013. Flaviviridae, in: Fields Virology. D. M. Knipe & P. M. Howley, Lippincott Williams & Wilkins, Philadelphia, pp. 712–746.

Lindlof, B., 1978. Aggressive dominance rank in relation to feeding by European hare. Viltrevy 10, 145–158.

Lindsay, D.S., Dubey, J.P., 2014. Toxoplasmosis in Wild and Domestic Animals, in: Toxoplasma Gondii. Elsevier, pp. 193–215.

Lopes, A.M., Capucci, L., Gavier-Widén, D., Le Gall-Reculé, G., Brocchi, E., Barbieri, I., Quéméner, A., Le Pendu, J., Geoghegan, J.L., Holmes, E.C., Esteves, P.J., Abrantes, J., 2014a. Molecular evolution and antigenic variation of European brown hare syndrome virus (EBHSV). Virology 468–470, 104–112. https://doi.org/10.1016/j.virol.2014.08.002

Lopes, A.M., Marques, S., Silva, E., Magalhães, M.J., Pinheiro, A., Alves, P.C., Le Pendu, J., Esteves, P.J., Thompson, G., Abrantes, J., 2014b. Detection of RHDV strains in the Iberian hare (Lepus granatensis): earliest evidence of rabbit lagovirus cross-species infection. Vet. Res. 45, 94. https://doi.org/10.1186/s13567-014-0094-7

Lucas, A., Bouley, G., Quinchon, C., Tocas, L., 1953. La Myxomatose Du Lievre. Bulletin De L'office International Des Epizooties 39, 770–776.

Luppi, M.M., Malta, M.C.C., Silva, T.M.A., Silva, F.L., Motta, R.O.C., Miranda, I., Ecco, R., Santos, R.L., 2008. Visceral leishmaniasis in captive wild canids in Brazil. Vet. Parasitol. 155, 146–151. https://doi.org/10.1016/j.vetpar.2008.04.024

Magallon, P., Bazin, J., 1953. La Myxomatose Du Lievre. Bulletin De L'office International Des Epizooties 39, 765–769.

Magistrali, C.F., Cucco, L., Pezzotti, G., Farneti, S., Cambiotti, V., Catania, S., Prati, P., Fabbi, M., Lollai, S., Mangili, P., Sebastiani, C., Bano, L., Dionisi, A.M., Luzzi, I., 2015. Characterisation of Yersinia pseudotuberculosis isolated from animals with yersiniosis during 1996–2013 indicates the presence of pathogenic and Far Eastern strains in Italy. Vet. Microbiol. 180, 161–166. https://doi.org/10.1016/j.vetmic.2015.08.020

Malta, M.C.C., Tinoco, H.P., Xavier, M.N., Vieira, A.L.S., Costa, E.A., Santos, R.L., 2010. Naturally acquired visceral leishmaniasis in non-human primates in Brazil. Vet. Parasitol. 169, 193–197. https://doi.org/10.1016/j.vetpar.2009.12.016

Mamuris, Z., Sfougaris, A.., Stamatis, C., 2001. Genetic structure of Greek brown hare (Lepus europaeus) populations as revealed by mtDBNA RFLP-PCR analysis: implications for conserving genetic diversity. Biol. Conserv. 101, 187–196. https://doi.org/10.1016/S0006-3207(01)00065-9

Marcato, P.S., Benazzi, C., Vecchi, G., Galeotti, M., Della Salda, L., Sarli, G., Lucidi, P., 1991. Clinical and pathological features of viral haemorrhagic disease of rabbits and the European brown hare syndrome. Rev. Sci. Tech. Int. Off. Epizoot. 10, 371–392.

Marco, I., Rosell, R., Cabezón, O., Beneria, M., Mentaberre, G., Casas, E., Hurtado, A., López-Olvera, J.R., Lavín, S., 2009. Serologic and virologic investigations into pestivirus infection in wild and domestic ruminants in the Pyrenees (NE Spain). Res. Vet. Sci. 87, 149–153. https://doi.org/10.1016/j.rvsc.2008.10.014

Marlier, D., Mainil, J., Linde, A., Vindevogel, H., 2000. Infectious agents associated with rabbit pneumonia: isolation of amyxomatous myxoma virus strains. Vet. J. Lond. Engl. 1997 159, 171–178. https://doi.org/10.1053/tvjl.1999.0413

Maubon, D., Ajzenberg, D., Brenier-Pinchart, M.-P., Dardé, M.-L., Pelloux, H., 2008. What are the respective host and parasite contributions to toxoplasmosis? Trends Parasitol. 24, 299–303. https://doi.org/10.1016/j.pt.2008.03.012

Maurin, M., Gyuranecz, M., 2016. Tularaemia: clinical aspects in Europe. Lancet Infect. Dis. 16, 113–124. https://doi.org/10.1016/S1473-3099(15)00355-2

Mazzei, M., Forzan, M., 2015. Detection of Hepatitis E Virus Antibodies in Domestic and Wild Animal Species in Central Italy. Clin. Microbiol. Open Access 04. https://doi.org/10.4172/2327-5073.1000227

Meng, X.J., Wiseman, B., Elvinger, F., Guenette, D.K., Toth, T.E., Engle, R.E., Emerson, S.U., Purcell, R.H., 2002. Prevalence of antibodies to hepatitis E virus in veterinarians working with swine and in normal blood donors in the United States and other countries. J. Clin. Microbiol. 40, 117–122.

Meyling, A., Houe, H., Jensen, A.M., 1990. Epidemiology of bovine virus diarrhoea virus. Rev. Sci. Tech. Int. Off. Epizoot. 9, 75–93.

Millán, J., Ferroglio, E., Solano-Gallego, L., 2014. Role of wildlife in the epidemiology of Leishmania infantum infection in Europe. Parasitol. Res. 113, 2005–2014. https://doi.org/10.1007/s00436-014-3929-2

Molina, R., Jiménez, M.I., Cruz, I., Iriso, A., Martín-Martín, I., Sevillano, O., Melero, S., Bernal, J., 2012. The hare (Lepus granatensis) as potential sylvatic reservoir of Leishmania infantum in Spain. Vet. Parasitol. 190, 268–271. https://doi.org/10.1016/j.vetpar.2012.05.006

Monaghan, P., Metcalfe, N.B., 1985. Group foraging in wild brown hares: effects of resource distribution and social status. Anim. Behav. 33, 993–999. https://doi.org/10.1016/S0003-3472(85)80033-6

Moreno, I., Álvarez, J., García, N., de la Fuente, S., Martínez, I., Marino, E., Toraño, A., Goyache, J., Vilas, F., Domínguez, L., Domínguez, M., 2014. Detection of anti-Leishmania infantum antibodies in sylvatic lagomorphs from an epidemic area of Madrid using the indirect immunofluorescence antibody test. Vet. Parasitol. 199, 264–267. https://doi.org/10.1016/j.vetpar.2013.10.010

Morillas-Marquez, F., Martin-Sanchez, J., Acedo-Sanchez, C., Pineda, J.A., Macias, J., Sanjuan-Garcia, J., 2002. Leishmania infantum (Protozoa, kinetoplastida): transmission from infected patients to experimental animal under conditions

that simulate needle-sharing. Exp. Parasitol. 100, 71–74. https://doi.org/10.1006/expr.2001.4678

Mörner, T., Sandström, G., Mattsson, R., Nilsson, P.O., 1988. Infections with Francisella tularensis biovar palaearctica in hares (Lepus timidus, Lepus europaeus) from Sweden. J. Wildl. Dis. 24, 422–433. https://doi.org/10.7589/0090-3558-24.3.422

Mustafa, M.L., Ayazi, E., Mohareb, E., Yingst, S., Zayed, A., Rossi, C.A., Schoepp, R.J., Mofleh, J., Fiekert, K., Akhbarian, Z., Sadat, H., Leslie, T., 2011. Crimean-Congo Hemorrhagic Fever, Afghanistan, 2009. Emerg. Infect. Dis. 17, 1940–1941. https://doi.org/10.3201/eid1710.110061

MYXOMATOSIS; OIE Terrestrial Manual , 2014.

Najdenski, H., Speck, S., 2012. Yersinia Infections, in: Gavier-Widén, D., Duff, J.P., Meredith, A. (Eds.), Infectious Diseases of Wild Mammals and Birds in Europe. Wiley-Blackwell, Oxford, UK, pp. 293–302. https://doi.org/10.1002/9781118342442.ch21

Nalca, A., Whitehouse, C.A., 2007. Crimean-Congo Hemorrhagic Fever Virus Infection among Animals, in: Ergonul, O., Whitehouse, C.A. (Eds.), Crimean-Congo Hemorrhagic Fever. Springer Netherlands, Dordrecht, pp. 155–165. https://doi.org/10.1007/978-1-4020-6106-6_13

Németh, V., Oldal, M., Egyed, L., Gyuranecz, M., Erdélyi, K., Kvell, K., Kalvatchev, N., Zeller, H., Bányai, K., Jakab, F., 2013. Serologic evidence of Crimean-Congo hemorrhagic fever virus infection in Hungary. Vector Borne Zoonotic Dis. Larchmt. N 13, 270–272. https://doi.org/10.1089/vbz.2012.1011

Oliva, G., Scalone, A., Foglia Manzillo, V., Gramiccia, M., Pagano, A., Di Muccio, T., Gradoni, L., 2006. Incidence and time course of Leishmania infantum infections examined by parasitological, serologic, and nested-PCR techniques in a cohort of naive dogs exposed to three consecutive transmission seasons. J. Clin. Microbiol. 44, 1318–1322. https://doi.org/10.1128/JCM.44.4.1318-1322.2006

Origgi, F.C., Pilo, P., 2016. *Francisella Tularensis* Clades B.FTN002-00 and B.13 Are Associated With Distinct Pathology in the European Brown Hare ( *Lepus*

*europaeus* ). Vet. Pathol. 53, 1220–1232. https://doi.org/10.1177/0300985816629718

Osterhaus, A. (Ed.), 1994. Virus infections of rodents and lagomorphs, Virus infections of vertebrates. Elsevier Science, Amsterdam ; New York.

Otter, A., 1996. Ovine abortion caused by Yersinia pseudotuberculosis. Vet. Rec. 138, 143–144.

Pace, D., 2014. Leishmaniasis. J. Infect. 69, S10–S18. https://doi.org/10.1016/j.jinf.2014.07.016

Paci, G., Lavazza, A., Ferretti, M., Santilli, F., Bagliacca, M., 2011. Relationship between Anti-European Brown Hare Syndrome Serological Titers and Brown Hare ( *Lepus europaeus* Pallas) Densities. Int. J. Zool. 2011, 1–5. https://doi.org/10.1155/2011/436193

Pangrazio, K.K., Costa, E.A., Amarilla, S.P., Cino, A.G., Silva, T.M.A., Paixão, T.A., Costa, L.F., Dengues, E.G., Diaz, A.A.R., Santos, R.L., 2009. Tissue distribution of Leishmania chagasi and lesions in transplacentally infected fetuses from symptomatic and asymptomatic naturally infected bitches. Vet. Parasitol. 165, 327–331. https://doi.org/10.1016/j.vetpar.2009.07.013

Papa, A., Mirazimi, A., Köksal, I., Estrada-Pena, A., Feldmann, H., 2015. Recent advances in research on Crimean-Congo hemorrhagic fever. J. Clin. Virol. 64, 137–143. https://doi.org/10.1016/j.jcv.2014.08.029

Papa, A., Sidira, P., Kallia, S., Ntouska, M., Zotos, N., Doumbali, E., Maltezou, H.C., Demiris, N., Tsatsaris, A., 2013. Factors associated with IgG positivity to Crimean-Congo hemorrhagic fever virus in the area with the highest seroprevalence in Greece. Ticks Tick-Borne Dis. 4, 417–420. https://doi.org/10.1016/j.ttbdis.2013.04.003

Papadogiannakis, E., Spanakos, G., Kontos, V., Menounos, P.G., Tegos, N., Vakalis, N., 2010. Molecular detection of Leishmania infantum in wild rodents (Rattus norvegicus) in Greece. Zoonoses Public Health 57, e23-25. https://doi.org/10.1111/j.1863-2378.2009.01264.x

Paton, N.I., Tee, N.W., Vu, C.K., Teo, T.P., 2001. Visceral abscesses due to Brucella suis infection in a retired pig farmer. Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. 32, E129-130. https://doi.org/10.1086/319748

Pavio, N., Meng, X.-J., Doceul, V., 2015. Zoonotic origin of hepatitis E. Curr. Opin. Virol. 10, 34–41. https://doi.org/10.1016/j.coviro.2014.12.006

Pikula, J., Beklova, M., Holesovska, Z., Skocovska, B., Treml, F., 2005. Ecology of brucellosis of the European hare in the Czech Republic. Vet. Med. – Czech 50, 105–109.

Poli, A., Mancianti, F., Marconcini, A., Cerri, D., Agrimi, P., 1987. Diseases of wild-living hares (Lepus europaeus P.) in Tuscany, in: Diseases of Wild-Living Hares (Lepus Europaeus P.) in Tuscany R. Ippen and H. D. Schroder (Eds.). Presented at the 29th international symposium of diseases of zoo animals, Akademie- Verlag, Berlin, Germany, pp. 341–346.

Posautz, A., Loncaric, I., Lundin, M., Hoffmann, D., Lavazza, A., Kelemen, Z., Beiglböck, C., Walzer, C., Kübber-Heiss, A., 2015. Health screening of free-ranging European brown hares (Lepus europaeus) on the German North-Sea island Pellworm. Acta Vet. Scand. 57. https://doi.org/10.1186/s13028-015-0132-0

Puggioni, G., Cavadini, P., Maestrale, C., Scivoli, R., Botti, G., Ligios, C., Le Gall-Reculé, G., Lavazza, A., Capucci, L., 2013. The new French 2010 Rabbit Hemorrhagic Disease Virus causes an RHD-like disease in the Sardinian Cape hare (Lepus capensis mediterraneus). Vet. Res. 44, 96. https://doi.org/10.1186/1297-9716-44-96

Quinnell, R.J., Courtenay, O., 2009. Transmission, reservoir hosts and control of zoonotic visceral leishmaniasis. Parasitology 136, 1915–1934. https://doi.org/10.1017/S0031182009991156

Richard, S., Oppliger, A., 2015. Zoonotic occupational diseases in forestry workers - Lyme borreliosis, tularemia and leptospirosis in Europe. Ann. Agric. Environ. Med. AAEM 22, 43–50. https://doi.org/10.5604/12321966.1141368

Rocchigiani, G., Ebani, V.V., Nardoni, S., Bertelloni, F., Bascherini, A., Leoni, A., Mancianti, F., Poli, A., 2018. Molecular survey on the occurrence of arthropod-borne pathogens in wild brown hares ( Lepus europaeus ) from Central Italy. Infect. Genet. Evol. 59, 142–147. https://doi.org/10.1016/j.meegid.2018.02.005

Ros Bascuñana, C., Nowotny, N., Belák, S., 1997. Detection and differentiation of rabbit hemorrhagic disease and European brown hare syndrome viruses by

amplification of VP60 genomic sequences from fresh and fixed tissue specimens. J. Clin. Microbiol. 35, 2492–2495.

Ruiz-Fons, F., Ferroglio, E., Gortázar, C., 2013. Leishmania infantum in free-ranging hares, Spain, 2004-2010. Euro Surveill. Bull. Eur. Sur Mal. Transm. Eur. Commun. Dis. Bull. 18, 20541.

Ruiz-Fons, F, Ferroglio, E., Gortázar, C., 2013. Leishmania infantum in free-ranging hares, Spain, 2004-2010. Euro Surveill. Bull. Eur. Sur Mal. Transm. Eur. Commun. Dis. Bull. 18, 20541.

Runge, M., von Keyserlingk, M., Braune, S., Voigt, U., Grauer, A., Pohlmeyer, K., Wedekind, M., Splettstoesser, W.D., Seibold, E., Otto, P., Müller, W., 2011. Prevalence of Francisella tularensis in brown hare (Lepus europaeus) populations in Lower Saxony, Germany. Eur. J. Wildl. Res. 57, 1085–1089. https://doi.org/10.1007/s10344-011-0522-1

Santiago, M.E.B., Vasconcelos, R.O., Fattori, K.R., Munari, D.P., Michelin, A. de F., Lima, V.M.F., 2007. An investigation of Leishmania spp. in Didelphis spp. from urban and peri-urban areas in Bauru (São Paulo, Brazil). Vet. Parasitol. 150, 283–290. https://doi.org/10.1016/j.vetpar.2007.09.026

Scherer, C.F.., Flores, E.., Weiblen, R., Caron, L., Irigoyen, L.., Neves, J.., Maciel, M.., 2001. Experimental infection of pregnant ewes with bovine viral diarrhea virus type-2 (BVDV-2): effects on the pregnancy and fetus. Vet. Microbiol. 79, 285–299. https://doi.org/10.1016/S0378-1135(00)00357-6

Schielke, A., Ibrahim, V., Czogiel, I., Faber, M., Schrader, C., Dremsek, P., Ulrich, R.G., Johne, R., 2015. Hepatitis E virus antibody prevalence in hunters from a district in Central Germany, 2013: a cross-sectional study providing evidence for the benefit of protective gloves during disembowelling of wild boars. BMC Infect. Dis. 15. https://doi.org/10.1186/s12879-015-1199-y

Scholz, H.C., Hubalek, Z., Sedlácek, I., Vergnaud, G., Tomaso, H., Al Dahouk, S., Melzer, F., Kämpfer, P., Neubauer, H., Cloeckaert, A., Maquart, M., Zygmunt, M.S., Whatmore, A.M., Falsen, E., Bahn, P., Göllner, C., Pfeffer, M., Huber, B., Busse, H.-J., Nöckler, K., 2008. Brucella microti sp. nov., isolated from the common vole Microtus arvalis. Int. J. Syst. Evol. Microbiol. 58, 375–382. https://doi.org/10.1099/ijs.0.65356-0

Scicluna, M.T., Lavazza, A., Capucci, L., 1994. European brown hare syndrome in northern Italy: results of a virological and serological survey. Rev. Sci. Tech. Int. Off. Epizoot. 13, 893–894.

Sedlák, K., Literák, I., Faldyna, M., Toman, M., Benák, J., 2000. Fatal toxoplasmosis in brown hares (Lepus europaeus): possible reasons of their high susceptibility to the infection. Vet. Parasitol. 93, 13–28.

Seleem, M.N., Boyle, S.M., Sriranganathan, N., 2010. Brucellosis: A re-emerging zoonosis. Vet. Microbiol. 140, 392–398. https://doi.org/10.1016/j.vetmic.2009.06.021

Serracca, L., Battistini, R., Rossini, I., Mignone, W., Peletto, S., Boin, C., Pistone, G., Ercolini, R., Ercolini, C., 2015. Molecular Investigation on the Presence of Hepatitis E Virus (HEV) in Wild Game in North-Western Italy. Food Environ. Virol. 7, 206–212. https://doi.org/10.1007/s12560-015-9201-9

Sert, H., Suchentrunk, F., Erdoğan, A., 2005. Genetic diversity within Anatolian brown hares (Lepus europaeus Pallas, 1778) and differentiation among Anatolian and European populations. Mamm. Biol. - Z. Für Säugetierkd. 70, 171–186. https://doi.org/10.1016/j.mambio.2004.10.003

Shepherd, A.J., Swanepoel, R., Cornel, A.J., Mathee, O., 1989. Experimental studies on the replication and transmission of Crimean-Congo hemorrhagic fever virus in some African tick species. Am. J. Trop. Med. Hyg. 40, 326–331.

Shepherd, A.J., Swanepoel, R., Shepherd, S.P., McGillivray, G.M., Searle, L.A., 1987. Antibody to Crimean-Congo hemorrhagic fever virus in wild mammals from southern Africa. Am. J. Trop. Med. Hyg. 36, 133–142.

Shimizu, K., 1958. Studies on toxoplasmosis 1. An outbreak of toxoplasmosis among hares (Lepus timidus ainu) in Sapporo. Jap. ]. Vet. Res 6.

Silva, F.L., Oliveira, R.G., Silva, T.M.A., Xavier, M.N., Nascimento, E.F., Santos, R.L., 2009. Venereal transmission of canine visceral leishmaniasis. Vet. Parasitol. 160, 55–59. https://doi.org/10.1016/j.vetpar.2008.10.079

Sjöstedt, A., 2007. Tularemia: history, epidemiology, pathogen physiology, and clinical manifestations. Ann. N. Y. Acad. Sci. 1105, 1–29. https://doi.org/10.1196/annals.1409.009

Slama, D., Haouas, N., Remadi, L., Mezhoud, H., Babba, H., Chaker, E., 2014. First detection of Leishmania infantum (Kinetoplastida: Trypanosomatidae) in Culicoides spp. (Diptera: Ceratopogonidae). Parasit. Vectors 7, 51. https://doi.org/10.1186/1756-3305-7-51

Smirnova, S.E., 1979. A comparative study of the Crimean hemorrhagic fever-Congo group of viruses. Arch. Virol. 62, 137–143.

Smith, D.B., Simmonds, P., members of the International Committee on the Taxonomy of Viruses Hepeviridae Study Group, Jameel, S., Emerson, S.U., Harrison, T.J., Meng, X.-J., Okamoto, H., Van der Poel, W.H.M., Purdy, M.A., 2014. Consensus proposals for classification of the family Hepeviridae. J. Gen. Virol. 95, 2223–2232. https://doi.org/10.1099/vir.0.068429-0

Smith, R.K., Vaughan Jennings, N., Harris, S., 2005. A quantitative analysis of the abundance and demography of European hares Lepus europaeus in relation to habitat type, intensity of agriculture and climate. Mammal Rev. 35, 1–24. https://doi.org/10.1111/j.1365-2907.2005.00057.x

Solano-Gallego, L., Koutinas, A., Miró, G., Cardoso, L., Pennisi, M.G., Ferrer, L., Bourdeau, P., Oliva, G., Baneth, G., 2009. Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. Vet. Parasitol. 165, 1–18. https://doi.org/10.1016/j.vetpar.2009.05.022

Solano-Gallego, L., Rossi, L., Scroccaro, A.M., Montarsi, F., Caldin, M., Furlanello, T., Trotta, M., 2012. Detection of Leishmania infantum DNA mainly in Rhipicephalus sanguineus male ticks removed from dogs living in endemic areas of canine leishmaniosis. Parasit. Vectors 5, 98. https://doi.org/10.1186/1756-3305-5-98

Souza, N.P., Almeida, A. do B.P.F. de, Freitas, T.P.T. de, Paz, R.C.R. da, Dutra, V., Nakazato, L., Sousa, V.R.F., 2010. [Leishmania (Leishmania) infantum chagasi in wild canids kept in captivity in the State of Mato Grosso]. Rev. Soc. Bras. Med. Trop. 43, 333–335.

Souza, T.D., Turchetti, A.P., Fujiwara, R.T., Paixão, T.A., Santos, R.L., 2014. Visceral leishmaniasis in zoo and wildlife. Vet. Parasitol. 200, 233–241. https://doi.org/10.1016/j.vetpar.2013.12.025

Spahr, C., Knauf-Witzens, T., Vahlenkamp, T., Ulrich, R.G., Johne, R., 2017. Hepatitis E virus and related viruses in wild, domestic and zoo animals: A review. Zoonoses Public Health. https://doi.org/10.1111/zph.12405

Spengler, J.R., Bergeron, É., Rollin, P.E., 2016. Seroepidemiological Studies of Crimean-Congo Hemorrhagic Fever Virus in Domestic and Wild Animals. PLoS Negl. Trop. Dis. 10, e0004210. https://doi.org/10.1371/journal.pntd.0004210

Spyrou, V., Stamatis, C., Birtsas, P., Psychas, V., Manolakou, K., Billinis, C., Mamuris, Z., 2013. Evidence for European brown hare syndrome virus introduction with translocated brown hares (Lepus europaeus): implications for management of restocking operations. Wildl. Res. 40, 545. https://doi.org/10.1071/WR12152

Ståhl, K., Alenius, S., 2012. BVDV control and eradication in Europe--an update. Jpn. J. Vet. Res. 60 Suppl, S31-39.

Stamatis, C., Suchentrunk, F., Moutou, K.A., Giacometti, M., Haerer, G., Djan, M., Vapa, L., Vukovic, M., Tvrtković, N., Sert, H., Alves, P.C., Mamuris, Z., 2009. Phylogeography of the brown hare ( *Lepus europaeus* ) in Europe: a legacy of south-eastern Mediterranean refugia? J. Biogeogr. 36, 515–528. https://doi.org/10.1111/j.1365-2699.2008.02013.x

Stamatis, C., Suchentrunk, F., Sert, H., Triantaphyllidis, C., Mamuris, Z., 2007. Genetic evidence for survival of released captive-bred brown hares Lepus europaeus during restocking operations in Greece. Oryx 41. https://doi.org/10.1017/S0030605307007132

Suchentrunk, F., Mamuris, Z., Sfougaris, A.I., Stamatis, C., 2003. Biochemical Genetic Variability in Brown Hares (Lepus europaeus) from Greece. Biochemical Genetics 41, 127–140.

Suckow, M.A., Stevens, K.A., Wilson, R.P. (Eds.), 2012. The laboratory rabbit, guinea pig, hamster, and other rodents, 1st ed. ed, American College of Laboratory Animal Medicine series. Academic Press/Elsevier, London ; Waltham, MA.

Syrjälä, P., Nylund, M., Heinikainen, S., 2005. European brown hare syndrome in free-living mountain hares (Lepus timidus) and European brown hares (Lepus europaeus) in Finland 1990-2002. J. Wildl. Dis. 41, 42–47. https://doi.org/10.7589/0090-3558-41.1.42

Tabak, F., Hakko, E., Mete, B., Ozaras, R., Mert, A., Ozturk, R., 2008. Is family screening necessary in brucellosis? Infection 36, 575–577. https://doi.org/10.1007/s15010-008-7022-6

Tantawi, H.H., Shony, M.O., Al-Tikriti, S.K., 1981. Antibodies to Crimean-Congo haemorrhagic fever virus in domestic animals in Iraq: a seroepidemiological survey. Int. J. Zoonoses 8, 115–120.

Tautz, N., Tews, B.A., Meyers, G., 2015. The Molecular Biology of Pestiviruses, in: Advances in Virus Research. Elsevier, pp. 47–160. https://doi.org/10.1016/bs.aivir.2015.03.002

Teyssou, R., Morvan, J., Leleu, J.P., Roumegou, P., Goullin, B., Carteron, B., 1989. A propos d'un cas de brucellose humaine a Brucella suis biovar 2. Médecine Mal. Infect. 19, 160–161. https://doi.org/10.1016/S0399-077X(89)80221-5

Thompson, R.C.A., Kutz, S., Smith, A., 2009. Parasite Zoonoses and Wildlife: Emerging Issues. Int. J. Environ. Res. Public. Health 6, 678–693. https://doi.org/10.3390/ijerph6020678

Treml, F., Pikula, J., Bandouchova, H., Horakova, J., 2007. European brown hare as a potential source of zoonotic agents. Vet. Med. (Praha) 52, 451–456.

Treml, F., Pikula, J., Holešovská, Z., 2003. Prevalence of Leptospirosis Antibodies in the European Hare (Lepus europaeus Pall.) in the District of Břeclav. Acta Vet. Brno 72, 377–381. https://doi.org/10.2754/avb200372030377

Tsokana, C.N., Sokos, C., Giannakopoulos, A., Mamuris, Z., Birtsas, P., Papaspyropoulos, K., Valiakos, G., Spyrou, V., Lefkaditis, M., Chatzopoulos, D.C., Kantere, M., Manolakou, K., Touloudi, A., Burriel, A.R., Ferroglio, E., Hadjichristodoulou, C., Billinis, C., 2015. First evidence of Leishmania infection in European brown hare (Lepus europaeus) in Greece: GIS analysis and phylogenetic position within the Leishmania spp. Parasitol. Res. https://doi.org/10.1007/s00436-015-4749-8

Tuncer, P., Yesilbag, K., Alpay, G., Dincer, E., Girisgin, O., Aydin, L., Uyar, Y., Ozkul, A., 2014. Crimean-Congo Hemorrhagic Fever infection in domestic animals in Marmara region, Western Turkey 61, 49–53.

Van Campen, H., Ridpath, J., Williams, E., Cavender, J., Edwards, J., Smith, S., Sawyer, H., 2001. ISOLATION OF BOVINE VIRAL DIARRHEA VIRUS FROM A FREE-

RANGING MULE DEER IN WYOMING. J. Wildl. Dis. 37, 306–311. https://doi.org/10.7589/0090-3558-37.2.306

Vanantwerpen, G., Van Damme, I., De Zutter, L., Houf, K., 2014. Within-batch prevalence and quantification of human pathogenic Yersinia enterocolitica and Y. pseudotuberculosis in tonsils of pigs at slaughter. Vet. Microbiol. 169, 223–227. https://doi.org/10.1016/j.vetmic.2013.12.019

Vaughan, N., Lucas, E.-A., Harris, S., White, P.C.L., 2003. Habitat associations of European hares Lepus europaeus in England and Wales: implications for farmland management. J. Appl. Ecol. 40, 163–175. https://doi.org/10.1046/j.1365-2664.2003.00784.x

Velarde, R., Cavadini, P., Neimanis, A., Cabezón, O., Chiari, M., Gaffuri, A., Lavín, S., Grilli, G., Gavier-Widén, D., Lavazza, A., Capucci, L., 2016. Spillover Events of Infection of Brown Hares ( *Lepus europaeus* ) with Rabbit Haemorrhagic Disease Type 2 Virus (RHDV2) Caused Sporadic Cases of an European Brown Hare Syndrome-Like Disease in Italy and Spain. Transbound. Emerg. Dis. https://doi.org/10.1111/tbed.12562

Verger, J.M., Grimont, F., Grimont, P.A., Grayon, M., 1987. Taxonomy of the genus Brucella. Ann. Inst. Pasteur Microbiol. 138, 235–238.

Vilček, š., Nettleton, P.F., 2006. Pestiviruses in wild animals. Vet. Microbiol. 116, 1–12. https://doi.org/10.1016/j.vetmic.2006.06.003

Weinberg, A.N., Branda, J.A., 2010. Case records of the Massachusetts General Hospital. Case 31-2010. A 29-year-old woman with fever after a cat bite. N. Engl. J. Med. 363, 1560–1568. https://doi.org/10.1056/NEJMcpc1007103

Westcott, D.G., Frossard, J.-P., Everest, D., Dastjerdi, A., Duff, J.P., Steinbach, F., Choudhury, B., 2014. Incursion of RHDV2-like variant in Great Britain. Vet. Rec. 174, 333. https://doi.org/10.1136/vr.g2345

Whitty, B.T., 1955. Myomatosis in the common hare. Irish Veterinary Journal 9, 267.

Wibbelt, G., Frölich, K., 2005. Infectious Diseases in European Brown Hare (Lepus europaeus). Wildl. Biol. Pract. 1. https://doi.org/10.2461/wbp.2005.1.11

Winkelmayer, R., Vodnansky, M., Paulsen, P., Gansterer, A., Treml, F., 2005. Explorative study on the seroprevalence of Brucella-, Francisella- and

Leptospira antibodies in the European hare (Lepus europaeus Pallas) of the Austrian - Czech border region. Vet. Med. Austria 92, 131–135.

Witte, S.T., Sponenberg, D.P., Collins, T.C., 1985. Abortion and early neonatal death of kids attributed to intrauterine Yersinia pseudotuberculosis infection. J. Am. Vet. Med. Assoc. 187, 834.

Wobeser, G., Campbell, G.D., Dallaire, A., McBurney, S., 2009. Tularemia, plague, yersiniosis, and Tyzzer's disease in wild rodents and lagomorphs in Canada: a review. Can. Vet. J. Rev. Veterinaire Can. 50, 1251–1256.

Woo, P.C.Y., Lau, S.K.P., Teng, J.L.L., Tsang, A.K.L., Joseph, M., Wong, E.Y.M., Tang, Y., Sivakumar, S., Xie, J., Bai, R., Wernery, R., Wernery, U., Yuen, K.-Y., 2014. New Hepatitis E Virus Genotype in Camels, the Middle East. Emerg. Infect. Dis. 20, 1044–1048. https://doi.org/10.3201/eid2006.140140

Woodroofe, G.M., Fenner, F., 1965. VIRUSES OF THE MYXOMA-FIBROMA SUBGROUP OF THE POXVIRUSES. I. PLAQUE PRODUCTION IN CULTURED CELLS, PLAQUE-REDUCTION TESTS, AND CROSS-PROTECTION TESTS IN RABBITS. Aust. J. Exp. Biol. Med. Sci. 43, 123–142.

Wunderink, H.F., Oostvogel, P.M., Frénay, I.H.M.E., Notermans, D.W., Fruth, A., Kuijper, E.J., 2014. Difficulties in diagnosing terminal ileitis due to Yersinia pseudotuberculosis. Eur. J. Clin. Microbiol. Infect. Dis. Off. Publ. Eur. Soc. Clin. Microbiol. 33, 197–200. https://doi.org/10.1007/s10096-013-1943-4

Wuthe, H.H., Aleksić, S., Kwapil, S., 1995. Yersinia in the European brown hare of northern Germany. Contrib. Microbiol. Immunol. 13, 51–54.

Yen, Y.C., Kong, L.X., Lee, L., Zhang, Y.Q., Li, F., Cai, B.J., Gao, S.Y., 1985. Characteristics of Crimean-Congo hemorrhagic fever virus (Xinjiang strain) in China. Am. J. Trop. Med. Hyg. 34, 1179–1182.

Yoshino, K., Ramamurthy, T., Nair, G.B., Fukushima, H., Ohtomo, Y., Takeda, N., Kaneko, S., Takeda, T., 1995. Geographical heterogeneity between Far East and Europe in prevalence of ypm gene encoding the novel superantigen among Yersinia pseudotuberculosis strains. J. Clin. Microbiol. 33, 3356–3358.

Zanet, S., Chiari, M., Battisti, E., Tizzani, P., Trisciuoglio, A., Taricco, L., Lavazza, A., Ferroglio, E., 2016. Leishmania infantum in wild lagomorphs: An epidemiological survey in Italy, in: Contributions to the 12th Conference of the

European Wildlife Disease Association (EWDA), Schumann, A., Wibbelt, G., Greenwood, A.D., Hofer, H., (Eds). Presented at the 12th Conference of the European Wildlife Disease Association (EWDA), Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany, p. 213.

Zheludkov, M.M., Tsirelson, L.E., 2010. Reservoirs of Brucella infection in nature. Biol. Bull. 37, 709–715. https://doi.org/10.1134/S106235901007006X

# CHAPTER I

# MOLECULAR DIAGNOSIS OF LEISHMANIASIS, SPECIES IDENTIFICATION AND PHYLOGENETIC ANALYSIS

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## Introduction

Leishmaniases are vector-borne infections caused by protozoa of genus *Leishmania*, affecting various mammals, mainly carnivores and humans. Clinical patent disease is relatively easy to be diagnosed and laboratory-confirmed by direct detection of the parasite in clinical samples. However, in subclinical cases detection of the causative agent is possible by highly sensitive diagnostic techniques such as molecular assays. Different molecular methods have been developed and evaluated including multilocus enzyme electrophoresis, conventional PCR based assays, quantitative Real Time PCR as well as simplified PCR methods.

More than 30 *Leishmania* species have been recognized, of which 20 are considered infective for humans and animals. The ability to distinguish between *Leishmania* species is crucial for differentiation of various forms of disease (Visceral, Cutaneous, Mucocutaneus) at least in humans in order to establish correct diagnosis and prognosis of the disease as well as to support decision-making regarding application of the appropriate treatment protocols.

Available tools for species identification and phylogenetic analysis include DNA sequencing analysis, restriction fragment length polymorphism (RFLP) analysis, and PCR-fingerprinting techniques as well as novel methods such as multilocus sequence typing (MLST) and multilocus microsatellite typing (MLMT). MLST is regarded as the most powerful phylogenetic approach and will be a better alternative to MLEE in the future. Various studies showed that the same target genomic regions can be used to compare distances among species but also to evaluate genetic diversity within species. This review aims to critically present current molecular approaches for Leishmaniasis diagnosis, species identification and phylogenetic analysis.

## Molecular Diagnosis

PCR is being used for the diagnosis of parasitic diseases, including Leishmaniasis. PCR is considered to be the most sensitive and specific technique among the methods applied so far for the direct detection and identification of the causative agent. The procedure is rapid and can be applied to a variety of clinical samples. Regarding the efficacy of the assay, it depends on the target selected for amplification (conserved or variable target region), the number of the target copies, the extraction technique used, the biological

sample tested and the PCR protocol adapted or developed (Baneth and Aroch, 2008; Cortes et al., 2004).

The PCR-based assays are advantageous over immunological techniques such as ELISA and IFAT as host species specific reagents are not required. The increased PCR sensitivity over serology for the detection of infection is of great interest in certain cases such as in patients with cutaneous, muco-cutaneous Leishmaniasis (CL or MCL) and the immunocompromised ones (e.g. coinfected with HIV, under chemotherapy etc). The former have low or no concentrations of antibodies against *Leishmania* due to the localized character of the disease while the latter present limited antibody production both resulting in negative serological tests (Singh, 2006). In particular, in chronic CL patients, who constitute the greater diagnostic challenge due to their low parasite density, PCR assays for the detection of *Leishmania* DNA presented 100% sensitivity. Moreover, the fact that antibodies remain detectable for years after successful treatment makes the application of PCR a necessity(De Almeida Silva et al., 2006).

PCR has been also proved to be valuable in the diagnosis of post-kala-azar dermal Leishmaniasis (PKDL) (Osman et al., 1998a). Additionally, the detection of parasite DNA has been shown to be a useful prognostic marker for the disease relapse or the development of PKDL even after successful treatment outcome. (Osman et al., 1998b). Furthermore, persistent infection has been found in apparently healed scars from MCL patients (Schubach et al., 1998), the presence of *Leishmania* braziliensis was reported in patients previously treated by immunotherapy or patients being at different stages of treatment and in subjects who had never presented clinical manifestations but they had lived in endemic areas and migrated to nonendemic regions (Guevara et al., 1993). Moreover, several studies reported that PCR detects parasitaemia a few weeks before the appearance of clinical manifestations. The detection of asymptomatic infected humans contributes to the prevention of the sand fly infection and the transfusion-transmitted kala-azar especially for the patients that require multiple transfusions, at least in endemic areas (Martín-Sánchez et al., 2004; Singh, 2006).

Regarding Canine Leishmaniasis, PCR assays constitute useful tools in cases of clinically healthy dogs which harbour infection but may never develop clinical disease. As the PCR positive results indicate infection, these assays could contribute to the prevention

of the importation of infected clinically healthy dogs to nonendemic areas where infection may spread via local sand fly vectors and the transmission via blood transfusion (Solano-Gallego et al., 2009). Finally, the parasite detection is crucial in case of negative results obtained by serology. This discrepancy may be attributed due to the gap between infection and seroconversion, the transient presence of specific antibodies and the possibility for some infected dogs never to be seroconverted. In contrast, false positive results may be obtained due to the existence of anti-*Leishmania* antibodies for a considerable time after convalescence (Maia et al., 2009).

On the other hand, a positive PCR result in asymptomatic dogs cannot support decision-making regarding treatment as the parasite DNA may be present for a long time after the parasite has been cleared while also a single negative PCR result in a clinically suspected dog cannot rule out infection.

Along with the need for PCR assays simplification, there is also a demand for standardization and optimization due to the lack of a universal PCR assay for the diagnosis of Leishmaniasis (Deborggraeve et al., 2008). Most laboratories perform "in-house" PCR assays using different primer pairs, DNA targets and PCR protocols (Schallig and Oskam, 2002).

A variety of clinical samples have been used for the detection of *Leishmania* DNA such as whole blood, buffy coat, bone marrow, lymph node, spleen, conjunctival swabs (Ferreira et al., 2008; Strauss-Ayali et al., 2004) and other biological samples such liver, lung, heart, penis, vagina, testis, semen, uterus, placenta, kidney, intestine, milk and urine (Maia and Campino, 2008) and more recently nasal, ear and oral swabs (Ferreira et al., 2013; Lombardo et al., 2012). Bone marrow, lymph node, spleen and skin are the tissues presenting the highest sensitivity for the diagnosis of Canine Leishmaniasis (Maia et al., 2009; Manna et al., 2008). The same holds true for the non invasive sampling techniques using conjunctival swabs (Ferreira et al., 2013; Strauss-Ayali et al., 2004). Whole blood, buffy coat, urine and the other biological samples mentioned above have been shown to be less sensitive.

Several target sequences and different PCR protocols have been described for the detection of *Leishmania* DNA. The most frequently used amplification targets are the Kinetoplast DNA minicircle (kDNA) (Andresen et al., 1997; da Silva et al., 2004; Hu et al., 2000; Martín-Sánchez et al., 2002; Pal et al., 2004; Salotra et al., 2001) and the small

subunit ribosomal RNA (SSU rRNA) (Cruz et al., 2006a, 2002; Lachaud et al., 2000; Mathis and Deplazes, 1995). There are various gene targets which are also commonly used such as the internal transcriped spacer (ITS) of 18SRNA gene (Azmi et al., 2011; Leite et al., 2010; Roelfsema et al., 2011; Rotureau et al., 2006; Schönian et al., 2003a; Strauss-Ayali et al., 2004), the mini exon derived RNA genes (spliced ladder) (E. Bensoussan et al., 2006; Fernandes et al., 1994; Katakura et al., 1998; J. Marfurt et al., 2003; Jutta Marfurt et al., 2003; Roelfsema et al., 2011; Serin et al., 2007) and a repetitive DNA sequence (Lachaud et al., 2002; Minodier et al., 1997).

It is worth mentioning that variable and sometimes conflicting results have been reported by several studies evaluating PCR using different target sequences in different host tissues. These results have been mostly obtained from asymptomatic infected hosts while also the results may vary depending on the sampling technique, storage method and the PCR protocol employed (Baneth and Aroch, 2008) . Some indicative studies evaluating the most frequently used PCR targets in different tissues are summarized in Table 1.

Table 1. Evaluation of the most frequently used PCR targets in different tissues.

| Target | PCR product size (bp) | Tissue tested | Sensitivity % | Specificity % | References |
|---|---|---|---|---|---|
| kDNA | 120,297, 790,792 | WB,BM | 68.8-100 | 100 | (da Silva et al., 2004) (Hu et al., 2000) (Pal et al., 2004) (Salotra et al., 2001) |
| ssurRNA | 358, 603 | WB,BM | 72.2-97 | 100 | (Cruz et al., 2002) (Cruz et al., 2006b)(Mathis and Deplazes, 1995) (Lachaud et al., 2000) |
| ITS1 | 300-350 | BM,WB,SB,SS,DS,CS,CB,SA | 68-100 | 100 | (Schönian et al., 2003b) (Rotureau et al., 2006) (Azmi et al., 2011) (Roelfsema et al., 2011) (Leite et al., 2010) |
| Mini-exon | 378-450 | BM,WB,SB,LA,DB, GB | 53.8-89.7 | 100 | (Katakura et al., 1998) (J. Marfurt et al., 2003) (E. Bensoussan et al., 2006) (Roelfsema et al., 2011) |

BM: Bone marrow, WB: whole blood, SB: Skin biopsy, SS: Skin scrapings, DS: Dermal smear, SA: Skin aspirates LA: Lesion aspirates CS: Conjunctival swab, CB: Cultured biopsies, DB: Duodenal biopsy, GB: Gastric biopsy

Real time PCR (or quantitative PCR-qPCR), a molecular technique which has revolusionized the pathogens diagnosis, is considered to be the reference method for molecular diagnosis in the future.

In recent years, qPCR assays based either on SYBR Green or TaqMan chemistries have been developed and evaluated for the detection, quantification and even species differentiation of *Leishmania spp* in a variety of clinical samples showing high sensitivity and reprodubility (Nicolas et al., 2002; Schulz et al., 2003). qPCR is considered to be a helpful tool for *Leishmania* diagnosis, monitoring during therapy, development of new drugs and diagnostic tools, comparison of drug efficacy or prophylactic schemes, and for epidemiological studies. Regarding diagnosis of Leishmaniasis, the kinetic study of parasitemia in the immunocompromised hosts, the diagnosis of relapses and the quantification of the low parasitic load in asymptomatic patients are of great interest (Paiva-Cavalcanti et al., 2010).

The qPCR is highly sensitive especially at the lower parasite loads (Mary et al., 2004; Vitale et al., 2004), specific and reproducible offering the ability to monitor therapy and to prevent relapses. The applications mentioned above make qPCR an attractive alternative to conventional PCR in routine diagnosis (Paiva-Cavalcanti et al., 2010; Vitale et al., 2004). Some of the studies carried out so far and their findings regarding the detection threshold, sensitivity and specificity are summarized in Table 2.

Table 2. Detection threshold, sensitivity and specificity of qPCR using various targets in different tissues.

| Target | Tissue tested | Detection threshold | Sensitivity % | Specificity % | References |
|---|---|---|---|---|---|
| kDNA | BM, WB | 0.001 p/ r | | | (Francino et al., 2006) |
| kDNA | WB | 0.07 p/ r | 100 | 83.33 | (de Paiva Cavalcanti et al., 2009) |
| kDNA | BM, WB, LN, CS, S, L, LU,K, BC | 0.03 p/ r | | | (Galletti et al., 2011) |
| kDNA | WB | 0.004 p/ r | | | (Abbasi et al., 2013) |
| TRYP | BS | | 98.7 | 59.8 | (Khosravi et al., 2012) |
| ITS1 | WB, SB, S | 0.25 p/s | | | (Talmi-Frank et al., 2010) |

BM: Bone marrow, WB: whole blood, SB: Skin biopsy, CS: Conjunctival swab, LN: Lymph node, S: Spleen, L: Liver, LU: Lung, K: Kidney, BC: Buffy coat, BS: Biopsy specimen, p/r:parasites/reaction, p/s: parasite/sample TRYP: tryparedoxin peroxidase gene

Given that PCR is restricted to well equiped laboratory settings, and that there is a need for simplification of the PCR assay and a demand for standardization and optimization (Reithinger and Dujardin, 2006), the described tools below may represent a good alternative for rapid and simple diagnosis of Leishmaniasis in endemic areas and epidemiological studies (Deborggraeve et al., 2008; van der Meide et al., 2008).

Quantitative nucleic acid sequence-based amplification (QT-NASBA) has proven to be a very sensitive and specific assay in diagnostic microbiology which is based on the amplification of single-stranded RNA sequences. In fact, this technique detects RNA in a background of DNA (Schallig and Oskam, 2002). Several QT-NASBA assays have been developed for the detection of *Leishmania* parasites including QT-NASBA combined with electro-chemiluminescence (ECL) (van der Meide et al., 2008, 2005) and QT-

NASBA combined with oligochromatographic technology (OC) (Deborggraeve et al., 2008; Mugasa et al., 2010b) for the detection of NASBA products The QT-NASBA assays developed, are commonly based on amplification of single-stranded 18S ribosomal RNA sequences (Basiye et al., 2010; Deborggraeve et al., 2008; Saad et al., 2010; van der Meide et al., 2008, 2005). This target is considered to be highly efficient for the diagnosis Leishmaniasis as each parasite contains a large number of copies of the 18SrRNA gene (van Eys et al., 1992) while also the cytoplasm is assumed to contain approximately 104 rRNA copies (van Eys et al., 1992). Moreover the target is present in all *Leishmania* species and it does not vary between different species allowing high sensitivity and quantification of all species in a similar manner (Deborggraeve et al., 2008; van der Meide et al., 2008, 2005). However, this target shows high similarity with the 18S rRNA gene sequence of *Endotrypanum*, *Crithidia, Wallacein*a, and *Leptomonas* organisms which may result in false positive results especially in the case of immunocompromised patients (Deborggraeve et al., 2008). The fact that NASBA detects RNA, makes it a molecular tool of great importance for the measurement of viable parasites. As a consequence, its application makes possible the assessment of the efficacy of drug therapies, the prediction of treatment outcome and the monitoring of the emergence of drug resistance. As it is well known, the DNA is still detected for a long time after parasite death, thus making RNA a preferable amplification target for the demonstration of parasite viability (Reithinger and Dujardin, 2006; Schallig and Oskam, 2002; van der Meide et al., 2005). Moreover, when targeting RNA, the starting number of the template molecules is much higher resulting in increased assay sensitivity and decreased sample volume required (Reithinger and Dujardin, 2006). The latter, makes also QT-NASBA a highly sensitive assay as it is able to detect very low target levels on clinical samples.

Loop-mediated isothermal amplification (LAMP), a novel method of DNA amplification under isothermal condition (Notomi et al., 2000), has been developed to detect Trypanosoma species, malaria, tuberculosis and filaria (Khan et al., 2012). Recently a reverse transcriptase step has been developed to specifically amplify RNA so as to amplify RNA viruses such as HIV and avian influenza viruses and to increase the assay sensitivity (Adams et al., 2010). The recently developed LAMP seems to be a promising diagnostic tool. The results obtained from several studies are encouraging as this assay

is much faster than conventional or nested PCR , it may be applied in field conditions, it shows high specificity and sensitivity (Khan et al., 2012; Mori and Notomi, 2009; Nagamine et al., 2002; Notomi et al., 2000; Takagi et al., 2009; Verma et al., 2013).
In the context of a generalized effort for simplification of the parasite detection, assays including PCR-ELISA and PCR-OC have been developed and evaluated. Several studies reported that PCR-ELISA showed high sensitivity. In a study, PCR-ELISA in blood samples from HIV negative VL patients was evaluated and presented higher sensitivity (83.9% and 73.2%) and specificity (100% and 87.2%) than conventional PCR (De Doncker et al., 2005). Other investigators have also evaluated the use of the assay in blood samples from HIV co-infected VL patients and PCR-ELISA found to be highly sensitive (Costa et al., 1996; Martin-Sanchez et al., 2001; Martín-Sánchez et al., 2002). Basiye et al, reported that PCR-OC is highly sensitive for *Leishmania* diagnosis on blood samples from VL patients (sensitivity 96,4% and specificity 88.8%) compared to NASBA-OC which was shown to be more specific (specificity 100%) (Basiye et al., 2010). In another study the repeatability and reproducibility of the assay was studied and found to be 95.9% and 98.1% in purified nucleic acid specimens and 87.1% and 91.7% in blood specimens spiked with parasites respectively (Mugasa et al., 2010a).

## Species Identification

The species identification is useful in areas with various co-existing *Leishmania* species such as the southern Mediterranean Basin where CL is caused by *L. major*, *L. tropica* or *L.infantum* and South America where CL may be caused by *L.mexicana* and *L.amazonensis* as well as the species of the subgenus *L.(Viannia).* Regarding the areas where only one species is considered to be responsible for the disease, the species identification is an important tool for the differentiation between *Leishmania* species and lower trypanosomatids related to the monoxenous parasites of insects of the genera *Leptomonas* or *Herpetomonas* which are also considered to cause VL in Southern Europe, South America and in the Indian subcontinent. As far as it concerns the non-endemic areas, they seem to be at risk for parasite importation due to the increasing international travel and population migration (SchöNian et al., 2010)

In recent years, there has been great scientific interest in the development of molecular tools, based on PCR or other amplification techniques, for *Leishmania* parasites identification at species and even strain level. The molecular tools used, range from amplification and subsequent RFLP or DNA sequence analysis of multicopy targets or multigene families, including coding and non-coding regions, and PCR-fingerprinting techniques to the recently developed MLST and MLMT with different discriminatory power, sensitivity and specificity while also each one has its specific advantages and drawbacks (SchöNian et al., 2010). Additionally, in most cases, the level of polymorphism found with coding or repeated non-coding PCR-amplified sequences is not refined enough to distinguish between closely related strains while application of MLST and MLMT approaches may reveal important strain polymorphisms.

PCR assays amplifying the conserved region of kinetoplast minicircle DNA or SSU rDNA have been shown to be the most sensitive, but they are able to identify *Leishmania* parasites only to the generic and/or subgeneric level (E. Bensoussan et al., 2006; Lachaud et al., 2002; Schönian et al., 2003a; van Eys et al., 1992). However, the kDNA PCR-RFLP assay has been used as a molecular marker for *Leishmania* identification at strain level and found to be discriminative between closely related organisms such as *L.infantum* MON-1. In this case, PCR-RFLP of whole minicircle DNA, a highly polymorphic assay, has been applied for differentiation between recrudescence and re-infection (Morales et al., 2002, 2001) and for *L.infantum* strain typing (Botilde et al., 2006). However, the interpretation of the RFLP patterns is difficult as well as the comparison of the results obtained between laboratories (Botilde et al., 2006; SchöNian et al., 2010)

The targets used for species identification include the ribosomal internal transcribed spacer (ITS) (Cupolillo et al., 1995; Nasereddin et al., 2008; Schönian et al., 2003a); the mini-exon gene (J. Marfurt et al., 2003; Jutta Marfurt et al., 2003); repetitive nuclear DNA sequences (Piarroux et al., 1993); the glucose-6-phosphate dehydrogenase gene (Castilho et al., 2003); gp63 genes (Victoir et al., 1998); hsp70 genes(Fraga et al., 2010; Garcia et al., 2004); cytochrome b gene (Kato et al., 2005), 7SL RNA gene sequences (Zelazny et al., 2005).

Other PCR-based approaches used for *Leishmania* parasites identification at strain level include the sequences of cysteine protease B (cpb) gene (Garcia et al., 2005; Hide and

Bañuls, 2006; Oshaghi et al., 2009; Quispe-Tintaya et al., 2005), the gp63 (Garcia et al., 2005; Mauricio et al., 2001), the ITS1 (Cupolillo et al., 2003, 1994; Rotureau et al., 2006; Schönian et al., 2001), the mini exon (Mauricio et al., 2004) and the kinetoplast minicircles (Chicharro et al., 2002; Cortes et al., 2006; Laurent et al., 2007; Morales et al., 2001; Noyes et al., 1998).

The digestion of ITS1 PCR product with the restriction enzyme HaeIII can distinguish all medically relevant *Leishmania* species. However, almost identical RFLP patterns arise for the representatives of the *L. donovani* complex *(L. donovani* and *L.infantum)* or *L. braziliensis* complex (*L. braziliensis, L. guyanensis, L. panamensis, L. peruviana* etc.) with a great variety of restriction enzymes (Schönian et al., 2003a). According to Schönian et al, in such a case, the sequencing of the ITS1 PCR product will allow the species differentiation (SchöNian et al., 2010). Nasereddin et al developed a simple reverse line blot hybridization (RLB) assay based on ITS1 sequences, which could distinguish all Old World *Leishmania* species, even *L. donovani* from *L.infantum*. This approach was found to be highly sensitive, approximately 10- to100-fold more sensitive than ITS1 PCR while the results obtained were comparable to those found by kDNA PCR (Nasereddin et al., 2008). Moreover, Talmi-Frank et al, described a new application of high resolution melt (HRM) analysis of a real time PCR product from the ITS1 region in samples from human, reservoir hosts and sand flies for rapid detection, quantification and speciation of Old World leishmanial species. In this assay, different characteristic high resolution melt analysis patterns were exhibited by *L.major*, *L.tropica*, *L. aethiopica*, and *L.infantum* making this approach able to distinguish all Old World *Leishmania* species causing human disease, except *L. donovani* from *L.infantum* (Talmi-Frank et al., 2010). Recently, an alternative technique, PCR-fluorescent fragment length analysis (PCR-FFL), has been developed by Tomás-Pérez et al, for use in *Leishmania* while its use has been reported previously for species identification in Trypanosoma (Hamilton et al., 2008; Tomas-Perez et al., 2013). In this study the fluorescently tagged primers used, were designed in the rRNA fragment ITS-1 and 7SL region. The amplified fragments were digested and their sizes were determined by an automated DNA sequencer. PCR-FFL was found to be accurate and more sensitive than PCR-RFLP analysis (Tomas-Perez et al., 2013)

Regarding the hsp70 PCR-RFLP approach, it is considered to be useful for the *L.(Viannia)* species discrimination while its sensitivity is poor for *L.(Leishmania)* species. Diagnostic

RFLP patterns for the *L.guyanensis* species complex as well as for *L. lainsoni* and *L. shawi* are produced after restriction with the enzyme HaeIII (da Silva et al., 2010; Garcia et al., 2004). However, this assay was not able to discriminate between *L. braziliensis* and *L. peruviana* as well as *L. naiffi*, requiring a second restriction enzyme for the differentiation (da Silva et al., 2010) while also *L. guyanensis* and *L. panamensis* both belonging to the *L. guyanensis* complex share identical RFLP pattern (Fraga et al., 2010). The discrimination of the species mentioned above is of great significance due to the fact that even if *L. braziliensis* is considered to be the main causative agent of MCL (Lessa et al., 2007) other *L.(Viannia)* species are also suspected of causing MCL. Additionally, a differential response to antimonial treatment has been documented (Arevalo et al., 2007; Llanos-Cuentas et al., 2008; Thomaz-Soccol et al., 2000). This assay was suggested to be applicable on clinical samples (Garcia et al., 2007; Montalvo et al., 2010).

Montalvo et al, extended the use of the hsp70 PCR-RFLP for identification of Old World and additional New World species and improved resolution within New World species complexes (Montalvo et al., 2010). Recently, they developed an adequate and flexible toolbox which consists of one improved and three new PCR approaches based on hsp70 target amplification and subsequent RFLP, able to diagnose and identify the most medically relevant New and Old World *Leishmania* species. The new PCR variants were highly sensitive and specific and they presented improved amplification efficiency in clinical samples compared to hsp70 PCR described previously by Garcia et al (Garcia et al., 2004). The choice of the most suitable PCR among the four described, depends on factors like the origin of infection, the sympatry of species, the imported versus endemic pathology, the clinical presentation and the clinical sample (Montalvo et al., 2011).

Fernandes et al first developed a PCR approach based on mini exon gene (Fernandes et al., 1994) which was later adapted by Mauricio et al. In this study the mini-exon PCR-RFLP was compared with ITS1 PCR-RFLP. Both targets were shown to be able to identify the strains studied but mini exon was found to be more polymorphic than ITS1 whereas neither ITS1 nor mini -exon produced as many robust groups as gp63 based restriction analyses published before (Mauricio et al., 2004, 2001). Marfurt et al also developed a mini-exon PCR-RFLP assay (Jutta Marfurt et al., 2003). The pair of primers deriving from

the conserved region was able to amplify DNA from Old and New World *Leishmania* species while the diversity detected in the non transcribed spacers represented an informative phylogenetic marker. The digestion of the PCR products with one or two different restriction enzymes resulted in species-specific patterns allowing the species differentiation. Thus, they designed a mini-exon PCR-RFLP genotyping scheme, using different restriction enzymes. However, a single EaeI digest was informative enough for the speciation needed in clinical setting (Jutta Marfurt et al., 2003). Furthermore, the repetitive character of this template made it highly sensitive even when applied to clinical samples (J. Marfurt et al., 2003).

On the other hand, when Bensoussan et al compared three PCR assays (kDNA,ITS1 and mini exon used as targets) found that mini exon presented the lowest sensitivity (53.8%) and suggested that this discrepancy may be attributed to the examination of stored clinical samples collected on filter papers instead of fresh samples, the extraction or the purification technique (Esther Bensoussan et al., 2006).

Rocha et al also adapted the PCR approach of Fernades et al and compared four PCR assays (kDNA and mini exon used as targets) for the evaluation of New World *Leishmania* strains typing. Species belonging to the subgenus *Leishmania* were not amplified with the mini exon target and the author suggested that this difference probably resulted from intraspecific variation (Rocha et al., 2010).

Recently, in another study, ITS1 and mini exon targets were compared with 18SrRNA in terms of sensitivity and discriminatory power in clinical samples, under routine laboratory settings. A new pair of primers for mini exon target was designed due to the inability of the previous published primers to amplify the target in all clinical samples while also the protocol was slightly modified in order to achieve better diagnostic sensitivity. However, ITS1 was found to be more sensitive and practical than mini exon. In contrast, mini exon was again more polymorphic and revealed a great discriminatory power in *L.(Viannia)* subgenus (Roelfsema et al., 2011).

The *Leishmania donovani* complex is the causative agent of visceral Leishmaniasis, the most severe form of the disease. The discrimination between the representatives of *L.donovani* complex, *L.infantum* and *L.donovani*, is important as they are morphologically indistinguishable while also they are associated with different epidemiology, ecology and pathology as *L.donovani* is anthroponotic and *L.infantum* is

anthropozoonotic. Moreover, there are not discriminative markers to identify certain strains which status is questioned. Thus, the development of molecular tools capable of identifying diagnostic markers and allowing a better understanding of phylogenetic relationships is of great importance. In a study a PCR assay based on cysteine proteinase B (cpb) was developed which was able to differentiate between the two species. The cathepsin-1 proteases CPB which belong to the papain-like superfamily, clan CA and family C1, play an important role in the host protein destruction and evasion of the host immune response (Alexander et al., 1998; Hide and Bañuls, 2006). CPB enzymes are encoded by a tandem array located in a single locus. Mundodi et al, have compared a L. donovani strain and a *L.chagasi*(syn *L.infantum*) strain and revealed at least five tandemly arranged genes (Mundodi et al., 2002). Hide and Banuls, used the last repeats of the cluster (cpbE for *L.infantum* and cpbF for *L. donovani*) and designed a PCR assay able to differentiate the two species by their fragment length as *L.donovani* strains were characterized by a 741-bp product and *L.infantum* strains by a 702-bp product. This PCR assay did not generate amplification for other *Leishmania* species neither trypanosomatids. Although sensitive and specific in cultured parasites, the assay is not sensitive enough for diagnosis on clinical samples (Hide and Bañuls, 2006). The fact that the species discrimination is based on 39 bp difference in PCR product may cause problems in species identification when using normal agarose gel electrophoresis and where both species are not available for comparison. Thus, another cpb PCR assay was developed with subsequent digestion with DraIII which cuts the 741-bp amplicon of *L.donovani* into 400 and 341 bp and a PCR using a species-specific primer pair capable of amplifying a 317 bp of *L. donovani* whereas it did not amplify *L.infantum* (Oshaghi et al., 2009). Two cpb PCR-RFLP and one fluorogenic PCR assay for the molecular typing of *L.donovani* complex have been also developed and it was reported that the assays described were valid and informative for *Leishmania* typing in clinical samples (Quispe Tintaya et al., 2004; Quispe-Tintaya et al., 2005). Furthermore, a multilocus approach, using new and previously reported targets including cpb genes, was applied to neotropical isolates (*L.braziliensis, L.peruviana, L.guyanensis, L.lainsoni and L. amazonensis*) and was shown to be a highly robust method of distinguishing different strains (Garcia et al., 2005).

Real-time PCR is considered to be a useful, sensitive, accurate and rapid tool for detection, quantification and even genetic characterization of *Leishmania* parasites. A LightCycler RT-PCR assay based on fluorescence melting curve analysis of PCR products generated from the minicircles of kDNA was developed. This assay was able to detect and differentiate four Old World *Leishmania* species ( *L. major* was differentiated from *L. donovani* and from *L.tropica* and *L.infantum*) (Nicolas et al., 2002). In another study, a qPCR based on glucosephosphate isomerase (GPI) gene was able to discriminate between subgenus *Viannia* and the complexes *L.mexicana, L.donovani/infantum* and *L.major* (Wortmann et al., 2005). A qPCR based on glucose-6-phosphate dehydrogenase (g6pd) using either SYBR-Green or TaqMan probes has also been described. This assay was able to differentiate *L.braziliensis* from other *L. (Viannia)* species and from those of *L.(Leishmania*) (Castilho et al., 2008). Weirather et al used a set of primers and probes for serial qPCR assays based on kDNA which was able to detect and differentiate *Leishmania* species in clinical samples due to different melt temperature of the amplicon or by observing the presence or absence of some amplicons (Weirather et al., 2011). Recently**,** tryparedoxine peroxidase gene was used as amplification target in a qPCR assay able to identify Old-World *Leishmania* species causing CL (Khosravi et al., 2012). An alternative 18S rDNA based qPCR using fluorescence resonance energy transfer probes (FRET) was able to discriminate the *L.donovani* complex, the *L.brasiliensis* complex, and species other than these based on the distinct melting temperature obtained (Schulz et al., 2003). Finally, a new q PCR assay based on FRET technology and melting curve analysis was designed based on mannose phosphate isomerase (MPI) and 6-phosphogluconate dehydrogenase (6PGD) genes which found to be highly sensitive and discriminative for the five species of *Leishmania* being evaluated (*L.braziliensis, L.panamensis, L.guyanensis, L.peruviana* and *L.lainsoni*) (Tsukayama et al., 2013).

Multilocus Enzyme Electrophoresis (MLEE), the technique which is regarded as the 'gold standard' for the identification of *Leishmania* parasites to species and subspecific levels and for genetic diversity studies, has been widely used since its introduction (Rioux et al., 1990a). MLEE detects different alleles of housekeeping genes indirectly by scoring the electrophoretic mobility of the enzymes they encode. The nucleotide differences in the genes encoding the enzymes are reflected by their mobility

differences. Thus, the parasites are identified by their enzymatic profile and are grouped in taxonomic units termed zymodemes, each one of whom consists of all the strains showing exactly the same profiles for all the enzymatic systems under study. Distinct combinations of isoenzyme mobilities for up to 15 enzymes have been assigned zymodeme numbers (MON-1–MON-274) (Bañuls et al., 2007).

However, this molecular method presents several disadvantages including the need for mass culture of *Leishmania* parasites and large amount of protein, it is timeconsuming, labour-intensive, costly and technically demanding. It is also worth mentioning that the MLEE methods used in Europe and in South America are based on different enzyme panels and cannot be compared directly (Cupolillo et al., 1994; Rioux et al., 1990a; SchöNian et al., 2010). As far as it concerns its discriminatory power, it is considered to be poor due to its inability to detect nucleotide substitutions that do not change the amino acid composition and changes in the amino acid composition that does not modify the electrophoretic mobility. The discriminatory power of MLEE for classifications below species level is limited. For instance, the *L.infantum* zymodeme MON1, the causative agent of the majority of visceral leishmaniasis cases around the Mediterranean basin and South America, has been shown to be genetically heterogeneous and polyphyletic with molecular markers presenting higher resolution level (Hide et al., 2001; Ochsenreither et al., 2006). Moreover, other molecular studies do not always agree with the classification of *Leishmania* parasites by MLEE. For instance, the differentiation between the representatives of *L.donovani* complex, *L.donovani* and *L.infantum*, is based on only one enzymatic system (glutamate–oxaloacetate transaminase-GOT) making the species distinction poor. In fact, the zymodeme MON-30 which was regarded as *L.infantum*, has recently shown to be *L.donovani* (Jamjoom et al., 2004; Zemanová et al., 2004). Furthermore, the existence of *L.archibaldi* as a distinct species belonging to *L.donovani* complex was supported by MLEE but it was not in agreement with the results of many different molecular markers (Lukes et al., 2007) while also *L.killicki* was not confirmed to be a separate species (Schönian et al., 2001; Schwenkenbecher et al., 2006) and *L. donovani* zymodeme MON-37 was assigned to strains of different genetic background (Mohammad Zahangir Alam et al., 2009; Bañuls et al., 2007; SchöNian et al., 2010).However, the codominant character of this molecular tool is advantageous as it is able to identify heterozygous

profiles and thus potential hybrids while also if the proteins are highly polymeric, the distinction can be made between a heterozygous profile and a mixed infection (Bañuls et al., 2007).

Randomly Amplified Polymorphic DNA (RAPD), a simple process, distinct from the PCR, based in the amplification of genomic DNA with short oligonucleotides of arbitrary nucleotide sequence used as primers, has been also applied for *Leishmania* spp. The primers are designed and used for the detection of polymorphisms without relying on prior knowledge of the DNA sequence to be amplified (Williams et al., 1990). From the advent of RAPD technique (Welsh and McClelland, 1990; Williams et al., 1990) numerous studies, only a few of them can be cited here, have been published reporting the use of RAPD as a molecular tool for *Leishmania* species identification and strain characterization. RAPD has been used for the investigation of the genomic diversity of *L.braziliensis* strains (Baptista et al., 2009; Gomes et al., 1995, p. 199), *L.major* isolates (Mahmoudzadeh-Niknam et al., 2012), *L.donovani* complex (Hamad et al., 2010; Mauricio et al., 1999; Zemanová et al., 2004) and *L.infantum* (Botilde et al., 2006; Hide et al., 2001; Segatto et al., 2012). Regarding the use of RAPD in species identification, it has been applied for the differentiation between the species *L.braziliensis, L.mexicana, L.infantum, L.tropica, L.chagasi, L.amazonensis and L.major* (Martinez et al., 2003), the identification and differentiation of Old World species at complex level (Ikram et al., 2002) and recently for the characterization of clinical isolates responsible for kala-azar in India (Khanra et al., 2011).

The main disadvantages of this technique are the need for parasite culture due to the use of non *Leishmania* specific primers and the poor reproducibility of the assay. Moreover, the bands of equal electrophoretic mobility may not be homologous and it is impossible to distinguish homozygous from heterozygous genotypes at specific loci because it is difficult to recognize allelic variants of randomly amplified polymorphic DNA markers in the absence of crossing data (Bañuls et al., 2007; SchöNian et al., 2010)

PCR hybridization is one of the first molecular methods for species identification and genotyping... DNA probes have been designed for *Leishmania* species identification. The most common target used for *Leishmania spp* identification is kDNA. DNA probes targeting kDNA have been applied for *L.major* (Smith et al., 1989), *L.infantum* (Gramiccia et al., 1992), *L.aethiopica* (Laskay et al., 1991), *L.mexicana* and *L.braziliensis*

(Rodríguez et al., 1994), and *L.mexicana*, *L.donovani* and *L.braziliensis* complexes (Brenière et al., 1999). Other specific probes developed include a cDNA probe, designed from a repetitive degenerate sequence isolated from *L.donovani*, which specifically hybridized only with isolates of the *L.donovani* complex (Howard et al., 1991) and two probes, the pDK10 and the pDK20, which were able to differentiate between the Old World *Leishmania* species belonging to *L.donovani* complex and between all Old World *Leishmania* species respectively (van Eys et al., 1991, 1989). DNA probes generated from mini-exon genes have also been developed (Grisard et al., 2000). Other probes developed so far include a *L.braziliensis* specific probe (Rodriguez et al., 1997) and *L.guyanensis*specific one (Rodriguez et al., 2000).

MLEE has been recently modified in a direct sequencing alleles detection method at each locus, called MLST. Partial sequences of approximately 700 bp in size, belonging to a defined set of housekeeping genes, are directly compared; the alleles are scored as identical or not and the same allele combinations are referred as sequence types. Alternatively, data analysis by sequencing of the alleles may be implemented. This technique was first used for bacterial pathogens whereas in *Leishmania*, steps have been taken to develop a MLST system. (Schönian et al., 2008)

The *L. donovani* complex has been studied by 2 sets of 5 loci for genes coding for enzymes used in MLEE (Mauricio et al., 2006; Zemanová et al., 2007). These 10 targets in combination should be a complete MLST system for application in *L. donovani* complex. These studies showed that results from MLST are in agreement with results from MLEE whereas some discrepancies were found and MLST presented higher resolution level such as a silent Single Nucleotide Polymorphism (SNP) in gpi that distinguishes between strains of *L.infantum* (Mauricio et al., 2006). Moreover, SNPs resulting in amino acid changes were also found in genes coding for enzymes giving indistinguishable electrophoretic profiles such as in nh2, which has the same protein band size for all *L.donovani* complex strains. These authors reported that MLST could be applied directly to clinical samples or to small-volume cultures. Furthermore, it can be used to detect recombination indirectly and for population genetics studies (Mauricio et al., 2006). Tsukayama et al investigated the intraspecific and interspecific variation in the coding sequences of four enzymes (gpi, mdh, mpi and 6pgd), used in the MLEE typing method, in order to identify SNPs able to discriminate among closely

related species. The assay was applied to clinical samples and successfully identified the species of *Leishmania* responsible for the clinical disease (Tsukayama et al., 2009). However, the analysis did not include sufficient diversity of strains for each species (SchöNian et al., 2010). Recently, in another study a combination of the previous published enzyme-coding genes (fh, g6pdh, icd, mpi and pgd) was used so as to differentiate the Chinese *Leishmania* isolates and to investigate their phylogenetic relationships (Zhang et al., 2013).

Multilocus microsatellite typing (MLMT) is based on the amplification of microsatellites sequences, tandem repeats of a simple nucleotide motif, 1-6 nucleotides, which are distributed abundantly in the eukaryotic and prokaryotic genomes and may reveal important strain polymorphisms. These markers are very useful for studying genetic variation between closely related organisms. Length polymorphisms in microsatellites sequences result from gain and loss of single repeat units which can be detected after amplification with specific to their flanking regions primers.

MLMT approaches developed so far for *Leishmania spp*, make use of sets of 14–20 unlinked microsatellite loci. Microsatellite loci with high discriminatory power and being suitable for characterizing closely related strains have been published for the *L.donovani* complex (M. Z. Alam et al., 2009; Gouzelou et al., 2012; M B Jamjoom et al., 2002; Kuhls et al., 2007), *L.donovani* strains (Mohammad Zahangir Alam et al., 2009) *L.major* (M. B. Jamjoom et al., 2002), *L.tropica* (Azmi et al., 2012; Schwenkenbecher et al., 2006) and for species of the subgenus *L. (Viannia)* (Oddone et al., 2009). Moreover, as the genetic diversity of *L.infantum* strains has been the subject of intense interest, several studies used MLMT approaches for the evaluation of the genomic variation in *L.infantum* strains (Ochsenreither et al., 2006; Segatto et al., 2012). It is worth mentioning that when MLMT was compared with other molecular markers for strain typing of *L.infantum*, the results obtained with kDNA PCR-RFLP were comparable to MLMT. kDNA and MLMT presented the highest discriminatory power especially for the MON-1 strains discrimination and appeared to be the most adequate for strain fingerprinting. However, MLMT is advantageous over kDNA PCR-RFLP because of its better reproducibility and feasibility of inter-lab comparisons and the co-dominant character of the markers used, making MLMT suitable for population genetic studies (Botilde et al., 2006). MLMT is suitable for high-throughput analysis and the data

obtained are reproducible and exchangeable between laboratories. Moreover, accurate, quality-controlled microsatellite profiles can be stored in databases and compared between different laboratories. In contrast to MLEE, selection does not seem to act on polymorphisms in microsatellite length while also the codominant nature of these markers permits the detection of the allelic variants. MLMT can be used directly on biological samples without prior culture of the parasite. DNA extracted from specimens spotted on filter paper or glass slides or from old Giemsa stained microscope slides was successfully applied in MLMT approaches (M. Z. Alam et al., 2009). It is recommended to use a panel of 10–20 unlinked microsatellite markers in all studies for nearly every species because microsatellite sequences are prone to homoplasy. Additionally, polymorphic repeats are not conserved between different species of *Leishmania* (M B Jamjoom et al., 2002; Ochsenreither et al., 2006; SchöNian et al., 2010).

## Phylogenetic Analysis

Phylogenetics is the study of evolutionary relationships among various groups of organisms (e.g., species or populations). Their relatedness is evaluated through morphological and molecular sequencing data. This analysis leads to a hypothesis about the evolutionary history of taxonomic groups, their phylogeny. Regarding evolution, it is considered to be a branching process. Populations are altered with time and may split into separate branches, hybridize or be eliminated. The order in which evolutionary events are assumed to have occurred is revealed and may be visualized in a phylogenetic tree.

As mentioned before, MLEE is still regarded as the reference technique for the identification of *Leishmania* species and subspecies. The data obtained from MLEE were analyzed by phenetic and cladistic techniques and led to the construction of the first phylogenetic tree of the genus *Leishmania* (Rioux et al., 1990b). The latter, revealed the monophyletic origin of the genus *Leishmania* and its subdivision into two subgenera, the *L.(Leishmania)* and the *L.(Viannia*) subgenus. *L.(Leishmania*) included the Old World species and *L.mexicana* and complexes from the New World. *L.(Viannia*) subgenus was composed from the other New World species. As *Sauroleishmania* was considered to be a separate genus, the lizard species were not included in these

studies. MLEE has been applied to a great variety and amount of isolates in comparison to other molecular methods in the past 25 years, resulting in the current classification system (Cupolillo et al., 1994; Rioux et al., 1990b). Phylogenetics based on different molecular methods, has confirmed the previous suggested taxonomy of the genus *Leishmania* by MLEE. However, the existence of a larger number of species has been proposed.

PCR-based methods with subsequent RFLP or DNA sequence analysis of multicopy targets or multigene families, to the recently developed MLST and MLMT, have been applied for the identification of the *Leishmania* species being responsible for the disease and for epidemiological studies in different endemic regions, as well as for taxonomic, phylogenetic, and population genetic studies. These tools except from their enhanced sensitivity they are also able to distinguish *Leishmania* parasites at species and intraspecies level. As for phylogenetic studies, the sequence analysis of single-copy gene targets is preferred while also the recombination and the different mutation rates between lineages make the use of one gene less suitable for the phylogenic analysis of the Trypanosomatidae or its subgroups(Schönian et al., 2013).

Several DNA targets have been used to reveal the phylogeny of the *Leishmania* genus including single-copy genes encoding the catalytic polypeptide of DNA polymerase a (polA), the largest subunit of RNA polymerase II (rpoIILS) (Croan et al., 1997) and 7SL RNA (Zelazny et al., 2005), the ITS (Berzunza-Cruz et al., 2002; Dávila and Momen, 2000), the N-acetylglucosamine-1-phosphate transferase (NAGT) gene (Waki et al., 2007), the mitochondrial cytochrome b gene (cytb) (Asato et al., 2009), and most recently, sequences of the hsp70 subfamily (Fraga et al., 2010).

Sequence analysis of these targets led to the conclusion that the subgenera L. (Leishmania) and *L. (Viannia)* constitute distinct monophyletic clades and that species of the Old and New World are segregated within the *L. (Leishmania*) subgenus. *Sauroleishmania* species branched off between the *L.(Leishmania*) and *L.(Viannia)* subgenera as an independent taxon suggesting that lizard *Leishmania* might be derived from mammalian parasites (Croan et al., 1997) and that they should be regarded as a subgenus of *Leishmania* rather than an independent genus (Noyes et al., 1998). However, the fact that RNA and DNA polymerase genes presented higher evolution

rate in the lizard *Leishmania* than in the mammalian *Leishmania* species set into question the exact taxonomic position of lizard parasites (Croan et al., 1997).

In another study, Cupolillo et al. based on various molecular criteria, suggested the division of the genus *Leishmania* into two sections, *Euleishmania* and *Paraleishmania*. *Euleishmania* consisted of the subgenera *L.(Leishmania), L.(Sauroleishmania), and L.(Viannia). Paraleishmania* included *L. hertigi, L.deanei, L.colombiensis, L.equatorensis, L.herreri,* and strains of *Endotrypanum*. In the latter section, the parasites of hystricomorph rodents, *L.hertigi* and *L.deanei* and the remaining species that are mainly parasites of sloths were genetically different while strains of *Endotrypanum* formed a paraphyletic group (Cupolillo et al., 2000).

More recently Fraga et al. analyzed the phylogeny of the genus *Leishmania* based on the hsp70 gene. In this study the isolates and strains used, were of different geographic origins. The resulting phylogeny supported that the monophyletic genus *Leishmania* consisted of three distinct subgenera, the *L.(Leishmania), L.(Viannia), and L.(Sauroleishmania).* The obtained phylogeny supported the following eight species: *L.donovani*, *L.major*, *L.tropica*, *L.mexicana*, *L.lainsoni*, *L.naiffi, L.guyanensis* and *L.braziliensis*. In some of these species, subspecies were recognized including *L.donovani infantum, L.guyanensis panamensis, and L.braziliensis peruviana*. The so far recognized species *L.aethiopica, L.garnhami, and L.amazonensis* did not form monophyletic clusters (Fraga et al., 2010).

Several discrepancies were reported for the taxonomic status of species obtained by MLEE compared to DNA based sequences. It is worth mentioning that the existence of *L.chagasi* and *L. archibaldi* as distinct species, was not supported by any molecular analyses as *L.chagasi* cannot be distinguished from strains of *L.infantum* and should therefore be regarded as South American strains of *L.infantum* (Kuhls et al., 2011; Maurício et al., 2000) whereas *L. archibaldi* is not a valid species (M. B. Jamjoom et al., 2002; Lukes et al., 2007). Numerous molecular studies did not even support the monophyly of the two remaining species, *L.donovani* and *L.infantum* (Asato et al., 2009; Croan et al., 1997; Fraga et al., 2010). Therefore, it was proposed that *L.donovani* is the only species of the *L.donovani* complex (Fraga et al., 2010) while *L.donovani infantum* was recognized as subspecies. Regarding other geographically defined genetic groups within *L.donovani*, it was suggested that they could be delimited.

Furthermore, the status of *L.killicki* has been debated. MLEE analysis supported the classification of *L.killicki* as a separate species while other molecular methods proposed that it was identical to *L.tropica* (Asato et al., 2009; Schönian et al., 2001; Schwenkenbecher et al., 2006). At the same time, *L.tropica* clusters to a single branch with *L.aethiopica*, making it difficult to be distinguished by the most of the DNA-based phylogenies (Asato et al., 2009; Fraga et al., 2010; Zelazny et al., 2005). It was suggested that they may represent different subspecies of the species *L.tropica* which is however needed to be investigated with a larger number of strains.

Another discrepancy concerns the existence of the *L.mexicana* complex species. The strains of *L.mexicana* and *L.amazonensis* species are overpresented in DNA based phylogenies while only one *L. garnhami* strain was analysed in the hsp70 trees. In the latter study, none of these species could be distinguished as a monophyletic clade and *L.mexicana* was the only recognized species (Fraga et al., 2010). These results are in agreement with previous published studies (Croan et al., 1997; Spanakos et al., 2008; Yurchenko et al., 2006) whereas they are in contrast to others (Asato et al., 2009; Berzunza-Cruz et al., 2002; Zelazny et al., 2005). Thus, the *L.mexicana* complex should be investigated, including *L.venezuelensis* and *L.aristidesi* strains, in order to evaluate the species and subspecies constituting this complex.

The same holds true for the *L.braziliensis* complex species. Several molecular phylogenies including hsp70, RAPD and MLEE, supported the distinction of *L.peruviana* from other strains of *L.braziliensis* (Banũls et al., 1999; Fraga et al., 2010) and it was recognized as a subspecies in the *L.braziliensis* complex. However, this classification was questioned by a study using monoclonal antibodies (Grimaldi and Tesh, 1993) and another one analyzing the microsatellite variation (Oddone et al., 2009) which suggested that strains of *L.peruviana* were grouped together with strains of *L.braziliensis* from Peru and from the Acre State, a Brazilian region bordering Peru. The use of a sufficiently large number of strains from different areas of distribution is needed so as the taxonomic status of the repsesentatives of the *L.braziliensis* complex to be evaluated. Moreover, in different phylogenetic trees, strains of *L.guyanensis* and *L.panamensis* formed a monophyletic cluster which was then divided into two monophyletic subclusters. Thus, the existence of two subspecies within the species *L.guyanensis* was proposed. A possible explanation for these discrepancies reported in

different studies regarding the taxonomic status of both *L.peruviana* and *L.panamensis*, is the application of different molecular markers and the analysis of different strains. Several molecular methods including MLEE (Cupolillo et al., 1994), PCR-RFLP of ITSrDNA (Cupolillo et al., 1995) and PCR-RFLP and sequence analysis of the hsp70 gene (da Silva et al., 2010), were also suggested the inclusion of *L.shawi* in the *L. guyanensis* group. The same applies for *L.naiffi* whereas *L.lainsoni* was confirmed to be the most divergent species inside the *L.(Viannia)* subgenus (da Silva et al., 2010; Fraga et al., 2010).

Noyes et al. (2002) identified a parasite isolated from human cutaneous lesions. Both stains were analysed by MLEE and found to be identical to each other and distantly related to all other *Leishmania* species. The application of other molecular methods revealed a low support for both its position basal to all *Euleishmania* and its clustering with *L.enriettii*. Thus, it was suggested that this strain may cluster with *L.*(*Leishmania*) or *L.(Viannia)* or form a novel clade within the *Euleishmania* either with or without *L.enriettii* (Noyes et al., 2002).

Recently *Leishmania* species isolated from clinical samples from immunocompetent and immunosuppressed patients in Thailand (Suankratay et al., 2010; Sukmee et al., 2008) and a focal CL outbreak in Ghana (Villinski et al., 2008) were identified and named as *L.siamensis*. Furthermore, novel *Leishmania* species, genetically indistinguishable, were isolated from kangaroos, wallaroos, and wallabies, living in captivity in the Northern Territory of Australia, a region that was considered free of *Leishmania* parasites (Rose et al., 2004). Additionally, autochthonous cases of CL in German and Swiss horses and in a Swiss cow could not be classified neither as Old World nor New World *Leishmania* species while they were found to be most closely related to *L.siamensis* (Lobsiger et al., 2010; Müller et al., 2009).

Finally, two new *L.(Viannia)* species were described and named *L.lindenbergi* (Silveira et al., 2002) and *L.utingensis*. The last one was represented by only one sample isolated from a *Lutzomyia tuberculata* sand fly. Although the sequence analysis of single-copy gene targets has shown to be informative, the use of several independent genes displaying different evolutionary histories is preferable (Simpson et al., 2006). Such genes have applied in MLST and provided new insights on taxonomy and evolutionary history of *Leishmania*. MLST is currently considered the most powerful phylogenetic approach, it has been shown to have high discriminatory power, reproducibility and

transportability of results between laboratories. Thus far, there are 10 published MLST targets available for the *L.donovani* complex (Mauricio et al., 2006; Zemanová et al., 2007), most of which are also applicable to other Old World *Leishmania* (Miles et al., 2009) and 4 targets for the sub-genus *Leishmania (Viannia)* (Tsukayama et al., 2009). This should form a complete MLST system applicable to *Leishmania* parasites.

## Conclusions

Molecular methods have revolutionized the diagnosis of Leishmaniasis. A variety of target sequences has been used and evaluated in different clinical samples of parasite hosts. Regarding PCR based assays, they were found to be rapid, sensitive and discriminative at species or even strain level. However, the diagnosis of Leishmaniasis remains a scientific challenge. There is a gap between the scientific advances, diagnostics and management of *Leishmania* infections in the field which should be decreased and an urgent need for standardization, optimization and simplification of PCR based applications. In this context, there is a generalized effort to make these assays available mainly in endemic areas around the world which will have an impact in disease control.

The great scientific interest for species identification may be attributed to its great significance in prompt diagnosis and prognosis of the disease, decision making regarding treatment and control measures. Despite the abundance of the studies carried out and the molecular markers used so far, the species discrimination is still difficult in several closely related species. Thus, molecular tools with great discriminatory power are constantly developed, evaluated and improved.

Many molecular tools have been used for the Leishmania phylogeny and the definition of its taxonomy. However, evaluation of the phylogenetic relationships of Leishmania species is not an easy task. Moreover, there is a need for simplification of the classification and a meaningful nomenclature of Leishmania genus particularly for the clinicians. The recently developed MLST is likely to become the gold standard basis for taxonomy and identification of Leishmania.

## References

Abbasi, I., Aramin, S., Hailu, A., Shiferaw, W., Kassahun, A., Belay, S., Jaffe, C., Warburg, A., 2013. Evaluation of PCR procedures for detecting and quantifying Leishmania donovani DNA in large numbers of dried human blood samples from a visceral leishmaniasis focus in Northern Ethiopia. BMC Infect. Dis. 13, 153. https://doi.org/10.1186/1471-2334-13-153

Adams, E.R., Schoone, G.J., Ageed, A.F., Safi, S.E., Schallig, H.D.F.H., 2010. Development of a reverse transcriptase loop-mediated isothermal amplification (LAMP) assay for the sensitive detection of Leishmania parasites in clinical samples. Am. J. Trop. Med. Hyg. 82, 591–596. https://doi.org/10.4269/ajtmh.2010.09-0369

Alam, Mohammad Zahangir, Haralambous, C., Kuhls, K., Gouzelou, E., Sgouras, D., Soteriadou, K., Schnur, L., Pratlong, F., Schönian, G., 2009. The paraphyletic composition of Leishmania donovani zymodeme MON-37 revealed by multilocus microsatellite typing. Microbes Infect. Inst. Pasteur 11, 707–715. https://doi.org/10.1016/j.micinf.2009.04.009

Alam, M. Z., Kovalenko, D.A., Kuhls, K., Nasyrova, R.M., Ponomareva, V.I., Fatullaeva, A.A., Razakov, S.A., Schnur, L.F., SchöNian, G., 2009. Identification of the agent causing visceral leishmaniasis in Uzbeki and Tajiki foci by analysing parasite DNA extracted from patients' Giemsa-stained tissue preparations. Parasitology 136, 981. https://doi.org/10.1017/S0031182009006465

Alexander, J., Coombs, G.H., Mottram, J.C., 1998. Leishmania mexicana cysteine proteinase-deficient mutants have attenuated virulence for mice and potentiate a Th1 response. J. Immunol. Baltim. Md 1950 161, 6794–6801.

Andresen, K., Gasim, S., Elhassan, A.M., Khalil, E.A., Barker, D.C., Theander, T.G., Kharazmi, A., 1997. Diagnosis of visceral leishmaniasis by the polymerase chain reaction using blood, bone marrow and lymph node samples from patients from the Sudan. Trop. Med. Int. Health TM IH 2, 440–444.

Arevalo, J., Ramirez, L., Adaui, V., Zimic, M., Tulliano, G., Miranda-Verástegui, C., Lazo, M., Loayza-Muro, R., De Doncker, S., Maurer, A., Chappuis, F., Dujardin, J.-C., Llanos-Cuentas, A., 2007. Influence of Leishmania (Viannia) species on the

response to antimonial treatment in patients with American tegumentary leishmaniasis. J. Infect. Dis. 195, 1846–1851. https://doi.org/10.1086/518041

Asato, Y., Oshiro, M., Myint, C.K., Yamamoto, Y., Kato, H., Marco, J.D., Mimori, T., Gomez, E.A.L., Hashiguchi, Y., Uezato, H., 2009. Phylogenic analysis of the genus Leishmania by cytochrome b gene sequencing. Exp. Parasitol. 121, 352–361. https://doi.org/10.1016/j.exppara.2008.12.013

Azmi, K., Nasereddin, A., Ereqat, S., Schnur, L., Schonian, G., Abdeen, Z., 2011. Methods incorporating a polymerase chain reaction and restriction fragment length polymorphism and their use as a 'gold standard' in diagnosing Old World cutaneous leishmaniasis. Diagn. Microbiol. Infect. Dis. 71, 151–155. https://doi.org/10.1016/j.diagmicrobio.2011.06.004

Azmi, K., Schnur, L., Schonian, G., Nasereddin, A., Pratlong, F., El Baidouri, F., Ravel, C., Dedet, J.-P., Ereqat, S., Abdeen, Z., 2012. Genetic, serological and biochemical characterization of Leishmania tropica from foci in northern Palestine and discovery of zymodeme MON-307. Parasit. Vectors 5, 121. https://doi.org/10.1186/1756-3305-5-121

Baneth, G., Aroch, I., 2008. Canine leishmaniasis: a diagnostic and clinical challenge. Vet. J. Lond. Engl. 1997 175, 14–15. https://doi.org/10.1016/j.tvjl.2006.11.011

Bañuls, A.-L., Hide, M., Prugnolle, F., 2007. Leishmania and the Leishmaniases: A Parasite Genetic Update and Advances in Taxonomy, Epidemiology and Pathogenicity in Humans, in: Advances in Parasitology. Elsevier, pp. 1–458.

Banũls, A.L., Hide, M., Tibayrenc, M., 1999. Molecular epidemiology and evolutionary genetics of Leischmania parasites. Int. J. Parasitol. 29, 1137–1147.

Baptista, C., Schubach, A.O., Madeira, M.F., Leal, C.A., Pires, M.Q., Oliveira, F.S., Conceição-Silva, F., Rosalino, C.M.V., Salgueiro, M.M., Pacheco, R.S., 2009. Leishmania (Viannia) braziliensis genotypes identified in lesions of patients with atypical or typical manifestations of tegumentary leishmaniasis: Evaluation by two molecular markers. Exp. Parasitol. 121, 317–322. https://doi.org/10.1016/j.exppara.2008.12.006

Basiye, F.L., Mbuchi, M., Magiri, C., Kirigi, G., Deborggraeve, S., Schoone, G.J., Saad, A.A., El-Safi, S., Matovu, E., Wasunna, M.K., 2010. Sensitivity and specificity of the Leishmania OligoC-TesT and NASBA-oligochromatography for diagnosis of

visceral leishmaniasis in Kenya. Trop. Med. Int. Health TM IH 15, 806–810. https://doi.org/10.1111/j.1365-3156.2010.02548.x

Bensoussan, E., Nasereddin, A., Jonas, F., Schnur, L.F., Jaffe, C.L., 2006. Comparison of PCR Assays for Diagnosis of Cutaneous Leishmaniasis. J. Clin. Microbiol. 44, 1435–1439. https://doi.org/10.1128/JCM.44.4.1435-1439.2006

Bensoussan, Esther, Nasereddin, A., Jonas, F., Schnur, L.F., Jaffe, C.L., 2006. Comparison of PCR assays for diagnosis of cutaneous leishmaniasis. J. Clin. Microbiol. 44, 1435–1439. https://doi.org/10.1128/JCM.44.4.1435-1439.2006

Berzunza-Cruz, M., Cabrera, N., Crippa-Rossi, M., Sosa Cabrera, T., Pérez-Montfort, R., Becker, I., 2002. Polymorphism analysis of the internal transcribed spacer and small subunit of ribosomal RNA genes of Leishmania mexicana. Parasitol. Res. 88, 918–925. https://doi.org/10.1007/s00436-002-0672-x

Botilde, Y., Laurent, T., Quispe Tintaya, W., Chicharro, C., Cañavate, C., Cruz, I., Kuhls, K., Schönian, G., Dujardin, J.-C., 2006. Comparison of molecular markers for strain typing of Leishmania infantum. Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis. 6, 440–446. https://doi.org/10.1016/j.meegid.2006.02.003

Brenière, S.F., Telleria, J., Bosseno, M.F., Buitrago, R., Bastrenta, B., Cuny, G., Banuls, A.L., Brewster, S., Barker, D.C., 1999. Polymerase chain reaction-based identification of New World Leishmania species complexes by specific kDNA probes. Acta Trop. 73, 283–293.

Castilho, T.M., Camargo, L.M.A., McMahon-Pratt, D., Shaw, J.J., Floeter-Winter, L.M., 2008. A real-time polymerase chain reaction assay for the identification and quantification of American Leishmania species on the basis of glucose-6-phosphate dehydrogenase. Am. J. Trop. Med. Hyg. 78, 122–132.

Castilho, T.M., Shaw, J.J., Floeter-Winter, L.M., 2003. New PCR assay using glucose-6-phosphate dehydrogenase for identification of Leishmania species. J. Clin. Microbiol. 41, 540–546.

Chicharro, C., Morales, M.A., Serra, T., Ares, M., Salas, A., Alvar, J., 2002. Molecular epidemiology of Leishmania infantum on the island of Majorca: a comparison of phenotypic and genotypic tools. Trans. R. Soc. Trop. Med. Hyg. 96 Suppl 1, S93-99.

Cortes, S., Mauricio, I., Almeida, A., Cristovão, J.M., Pratlong, F., Dedet, J.P., Campino, L., 2006. Application of kDNA as a molecular marker to analyse Leishmania infantum diversity in Portugal. Parasitol. Int. 55, 277–283. https://doi.org/10.1016/j.parint.2006.07.003

Cortes, S., Rolão, N., Ramada, J., Campino, L., 2004. PCR as a rapid and sensitive tool in the diagnosis of human and canine leishmaniasis using Leishmania donovani s.l.-specific kinetoplastid primers. Trans. R. Soc. Trop. Med. Hyg. 98, 12–17.

Costa, J.M., Durand, R., Deniau, M., Rivollet, D., Izri, M., Houin, R., Vidaud, M., Bretagne, S., 1996. PCR enzyme-linked immunosorbent assay for diagnosis of leishmaniasis in human immunodeficiency virus-infected patients. J. Clin. Microbiol. 34, 1831–1833.

Croan, D.G., Morrison, D.A., Ellis, J.T., 1997. Evolution of the genus Leishmania revealed by comparison of DNA and RNA polymerase gene sequences. Mol. Biochem. Parasitol. 89, 149–159.

Cruz, I., Cañavate, C., Rubio, J.M., Morales, M.A., Chicharro, C., Laguna, F., Jiménez-Mejías, M., Sirera, G., Videla, S., Alvar, J., Spanish HIV-Leishmania Study Group, 2002. A nested polymerase chain reaction (Ln-PCR) for diagnosing and monitoring Leishmania infantum infection in patients co-infected with human immunodeficiency virus. Trans. R. Soc. Trop. Med. Hyg. 96 Suppl 1, S185-189.

Cruz, I., Chicharro, C., Nieto, J., Bailo, B., Canavate, C., Figueras, M.-C., Alvar, J., 2006a. Comparison of New Diagnostic Tools for Management of Pediatric Mediterranean Visceral Leishmaniasis. J. Clin. Microbiol. 44, 2343–2347. https://doi.org/10.1128/JCM.02297-05

Cruz, I., Chicharro, C., Nieto, J., Bailo, B., Canavate, C., Figueras, M.-C., Alvar, J., 2006b. Comparison of New Diagnostic Tools for Management of Pediatric Mediterranean Visceral Leishmaniasis. J. Clin. Microbiol. 44, 2343–2347. https://doi.org/10.1128/JCM.02297-05

Cupolillo, E., Brahim, L.R., Toaldo, C.B., de Oliveira-Neto, M.P., de Brito, M.E.F., Falqueto, A., de Farias Naiff, M., Grimaldi, G., 2003. Genetic Polymorphism and Molecular Epidemiology of Leishmania (Viannia) braziliensis from Different Hosts and Geographic Areas in Brazil. J. Clin. Microbiol. 41, 3126–3132. https://doi.org/10.1128/JCM.41.7.3126-3132.2003

Cupolillo, E., Grimaldi, G.J., Momen, H., 1994. A general classification of New World Leishmania using numerical zymotaxonomy. Am. J. Trop. Med. Hyg. 50, 296–311.

Cupolillo, E., Grimaldi Júnior, G., Momen, H., Beverley, S.M., 1995. Intergenic region typing (IRT): a rapid molecular approach to the characterization and evolution of Leishmania. Mol. Biochem. Parasitol. 73, 145–155.

Cupolillo, E., Medina-Acosta, E., Noyes, H., Momen, H., Grimaldi, G.J., 2000. A revised classification for Leishmania and Endotrypanum. Parasitol. Today Pers. Ed 16, 142–144.

da Silva, E.S., Gontijo, C.M.F., Pacheco, R. da S., Brazil, R.P., 2004. Diagnosis of human visceral leishmaniasis by PCR using blood samples spotted on filter paper. Genet. Mol. Res. GMR 3, 251–257.

da Silva, L.A., de Sousa, C.D.S., da Graça, G.C., Porrozzi, R., Cupolillo, E., 2010. Sequence analysis and PCR-RFLP profiling of the hsp70 gene as a valuable tool for identifying Leishmania species associated with human leishmaniasis in Brazil. Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis. 10, 77–83. https://doi.org/10.1016/j.meegid.2009.11.001

Dávila, A.M., Momen, H., 2000. Internal-transcribed-spacer (ITS) sequences used to explore phylogenetic relationships within Leishmania. Ann. Trop. Med. Parasitol. 94, 651–654.

De Almeida Silva, L., Romero, H.D., Prata, A., Costa, R.T., Nascimento, E., Carvalho, S.F.G., Rodrigues, V., 2006. Immunologic tests in patients after clinical cure of visceral leishmaniasis. Am. J. Trop. Med. Hyg. 75, 739–743.

De Doncker, S., Hutse, V., Abdellati, S., Rijal, S., Singh Karki, B.M., Decuypere, S., Jacquet, D., Le Ray, D., Boelaert, M., Koirala, S., Dujardin, J.-C., 2005. A new PCR-ELISA for diagnosis of visceral leishmaniasis in blood of HIV-negative subjects. Trans. R. Soc. Trop. Med. Hyg. 99, 25–31. https://doi.org/10.1016/j.trstmh.2004.01.015

de Paiva Cavalcanti, M., Felinto de Brito, M.E., de Souza, W.V., de Miranda Gomes, Y., Abath, F.G.C., 2009. The development of a real-time PCR assay for the quantification of Leishmania infantum DNA in canine blood. Vet. J. Lond. Engl. 1997 182, 356–358. https://doi.org/10.1016/j.tvjl.2008.05.018

Deborggraeve, S., Laurent, T., Espinosa, D., Van der Auwera, G., Mbuchi, M., Wasunna, M., El-Safi, S., Al-Basheer, A.A., Arévalo, J., Miranda-Verástegui, C., Leclipteux, T., Mertens, P., Dujardin, J.-C., Herdewijn, P., Büscher, P., 2008. A simplified and standardized polymerase chain reaction format for the diagnosis of leishmaniasis. J. Infect. Dis. 198, 1565–1572. https://doi.org/10.1086/592509

Fernandes, O., Murthy, V.K., Kurath, U., Degrave, W.M., Campbell, D.A., 1994. Mini-exon gene variation in human pathogenic Leishmania species. Mol. Biochem. Parasitol. 66, 261–271.

Ferreira, S. de A., Almeida, G.G., Silva, S. de O., Vogas, G.P., Fujiwara, R.T., de Andrade, A.S.R., Melo, M.N., 2013. Nasal, oral and ear swabs for canine visceral leishmaniasis diagnosis: new practical approaches for detection of Leishmania infantum DNA. PLoS Negl. Trop. Dis. 7, e2150. https://doi.org/10.1371/journal.pntd.0002150

Ferreira, S. de A., Ituassu, L.T., de Melo, M.N., de Andrade, A.S.R., 2008. Evaluation of the conjunctival swab for canine visceral leishmaniasis diagnosis by PCR-hybridization in Minas Gerais State, Brazil. Vet. Parasitol. 152, 257–263. https://doi.org/10.1016/j.vetpar.2007.12.022

Fraga, J., Montalvo, A.M., De Doncker, S., Dujardin, J.-C., Van der Auwera, G., 2010. Phylogeny of Leishmania species based on the heat-shock protein 70 gene. Infect. Genet. Evol. 10, 238–245. https://doi.org/10.1016/j.meegid.2009.11.007

Francino, O., Altet, L., Sánchez-Robert, E., Rodriguez, A., Solano-Gallego, L., Alberola, J., Ferrer, L., Sánchez, A., Roura, X., 2006. Advantages of real-time PCR assay for diagnosis and monitoring of canine leishmaniosis. Vet. Parasitol. 137, 214–221. https://doi.org/10.1016/j.vetpar.2006.01.011

Galletti, E., Bonilauri, P., Bardasi, L., Fontana, M.C., Ramini, M., Renzi, M., Dosa, G., Merialdi, G., 2011. Development of a minor groove binding probe based real-time PCR for the diagnosis and quantification of Leishmania infantum in dog specimens. Res. Vet. Sci. 91, 243–245. https://doi.org/10.1016/j.rvsc.2011.01.004

Garcia, A.L., Kindt, A., Quispe-Tintaya, K.W., Bermudez, H., Llanos, A., Arevalo, J., Bañuls, A.L., De Doncker, S., Le Ray, D., Dujardin, J.C., 2005. American

tegumentary leishmaniasis: antigen-gene polymorphism, taxonomy and clinical pleomorphism. Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis. 5, 109–116. https://doi.org/10.1016/j.meegid.2004.07.003

Garcia, A.L., Parrado, R., De Doncker, S., Bermudez, H., Dujardin, J.-C., 2007. American tegumentary leishmaniasis: direct species identification of Leishmania in non-invasive clinical samples. Trans. R. Soc. Trop. Med. Hyg. 101, 368–371. https://doi.org/10.1016/j.trstmh.2006.06.009

Garcia, L., Kindt, A., Bermudez, H., Llanos-Cuentas, A., De Doncker, S., Arevalo, J., Quispe Tintaya, K.W., Dujardin, J.-C., 2004. Culture-Independent Species Typing of Neotropical Leishmania for Clinical Validation of a PCR-Based Assay Targeting Heat Shock Protein 70 Genes. J. Clin. Microbiol. 42, 2294–2297. https://doi.org/10.1128/JCM.42.5.2294-2297.2004

Gomes, R.F., Macedo, A.M., Pena, S.D., Melo, M.N., 1995. Leishmania (Viannia) braziliensis: genetic relationships between strains isolated from different areas of Brazil as revealed by DNA fingerprinting and RAPD. Exp. Parasitol. 80, 681–687. https://doi.org/10.1006/expr.1995.1084

Gouzelou, E., Haralambous, C., Amro, A., Mentis, A., Pratlong, F., Dedet, J.-P., Votypka, J., Volf, P., Toz, S.O., Kuhls, K., Schönian, G., Soteriadou, K., 2012. Multilocus microsatellite typing (MLMT) of strains from Turkey and Cyprus reveals a novel monophyletic L. donovani sensu lato group. PLoS Negl. Trop. Dis. 6, e1507. https://doi.org/10.1371/journal.pntd.0001507

Gramiccia, M., Smith, D.F., Angelici, M.C., Ready, P.D., Gradoni, L., 1992. A kinetoplast DNA probe diagnostic for Leishmania infantum. Parasitology 105 ( Pt 1), 29–34.

Grimaldi, G.J., Tesh, R.B., 1993. Leishmaniases of the New World: current concepts and implications for future research. Clin. Microbiol. Rev. 6, 230–250.

Grisard, E.C., Steindel, M., Shaw, J.J., Ishikawa, E.A., Carvalho-Pinto, C.J., Eger-Mangrich, I., Toma, H.K., Lima, J.H., Romanha, A.J., Campbell, D.A., 2000. Characterization of Leishmania sp. strains isolated from autochthonous cases of human cutaneous leishmaniasis in Santa Catarina State, southern Brazil. Acta Trop. 74, 89–93.

Guevara, P., Ramírez, J.L., Rojas, E., Scorza, J.V., González, N., Añez, N., 1993. Leishmania braziliensis in blood 30 years after cure. Lancet 341, 1341.

Hamad, S.H., Khalil, E.A.G., Musa, A.M., Ibrahim, M.E., Younis, B.M., Elfaki, M.E.E., El-Hassan, A.M., Leishmaniasis Research Group, Sudan, 2010. Leishmania donovani: genetic diversity of isolates from Sudan characterized by PCR-based RAPD. Exp. Parasitol. 125, 389–393. https://doi.org/10.1016/j.exppara.2010.03.008

Hamilton, P.B., Adams, E.R., Malele, I.I., Gibson, W.C., 2008. A novel, high-throughput technique for species identification reveals a new species of tsetse-transmitted trypanosome related to the Trypanosoma brucei subgenus, Trypanozoon. Infect. Genet. Evol. 8, 26–33. https://doi.org/10.1016/j.meegid.2007.09.003

Hide, M., Bañuls, A.-L., 2006. Species-specific PCR assay for L. infantum/L. donovani discrimination. Acta Trop. 100, 241–245. https://doi.org/10.1016/j.actatropica.2006.10.012

Hide, M., Bañuls, A.L., Tibayrenc, M., 2001. Genetic heterogeneity and phylogenetic status of Leishmania (Leishmania) infantum zymodeme MON-1: epidemiological implications. Parasitology 123, 425–432.

Howard, M.K., Kelly, J.M., Lane, R.P., Miles, M.A., 1991. A sensitive repetitive DNA probe that is specific to the Leishmania donovani complex and its use as an epidemiological and diagnostic reagent. Mol. Biochem. Parasitol. 44, 63–72.

Hu, X.S., Yang, W.T., Lu, H.G., Yan, H.P., Cheng, J.P., Ma, Y., Jin, B.Q., Zhang, T., 2000. Sequencing a specific kinetoplast DNA fragment of Leishmania donovani for polymerase chain reaction amplification in diagnosis of leishmaniasis in bone marrow and blood samples. J. Parasitol. 86, 822–826. https://doi.org/10.1645/0022-3395(2000)086[0822:SASKDF]2.0.CO;2

Ikram, G., Dellagi, K., Ismaïl, R.B., 2002. Random amplified polymorphic DNA technique for identification and differentiation of old world Leishmania species. Am. J. Trop. Med. Hyg. 66, 152–156.

Jamjoom, M.B., Ashford, R.W., Bates, P.A., Chance, M.L., Kemp, S.J., Watts, P.C., Noyes, H.A., 2004. Leishmania donovani is the only cause of visceral leishmaniasis in East Africa; previous descriptions of L. infantum and “L. archibaldi” from this region are a consequence of convergent evolution in the isoenzyme data. Parasitology 129, 399–409. https://doi.org/10.1017/S0031182004005955

Jamjoom, M B, Ashford, R.W., Bates, P.A., Kemp, S.J., Noyes, H.A., 2002. Towards a standard battery of microsatellite markers for the analysis of the Leishmania donovani complex. Ann. Trop. Med. Parasitol. 96, 265–270. https://doi.org/10.1179/000349802125000790

Jamjoom, M. B., Ashford, R.W., Bates, P.A., Kemp, S.J., Noyes, H.A., 2002. Polymorphic microsatellite repeats are not conserved between Leishmania donovani and Leishmania major. Mol. Ecol. Notes 2, 104–106. https://doi.org/10.1046/j.1471-8286.2002.00161.x

Katakura, K., Kawazu, S., Naya, T., Nagakura, K., Ito, M., Aikawa, M., Qu, J.Q., Guan, L.R., Zuo, X.P., Chai, J.J., Chang, K.P., Matsumoto, Y., 1998. Diagnosis of kala-azar by nested PCR based on amplification of the Leishmania mini-exon gene. J. Clin. Microbiol. 36, 2173–2177.

Kato, H., Uezato, H., Katakura, K., Calvopiña, M., Marco, J.D., Barroso, P.A., Gomez, E.A., Mimori, T., Korenaga, M., Iwata, H., Nonaka, S., Hashiguchi, Y., 2005. Detection and identification of Leishmania species within naturally infected sand flies in the andean areas of ecuador by a polymerase chain reaction. Am. J. Trop. Med. Hyg. 72, 87–93.

Khan, M.G.M., Bhaskar, K.R.H., Salam, M.A., Akther, T., Pluschke, G., Mondal, D., 2012. Diagnostic accuracy of loop-mediated isothermal amplification (LAMP) for detection of Leishmania DNA in buffy coat from visceral leishmaniasis patients. Parasit. Vectors 5, 280. https://doi.org/10.1186/1756-3305-5-280

Khanra, S., Bandopadhyay, S.K., Chakraborty, P., Datta, S., Mondal, D., Chatterjee, M., Naskar, K., Roy, S., Manna, M., 2011. Characterization of the recent clinical isolates of Indian Kala-azar patients by RAPD-PCR method. J. Parasit. Dis. Off. Organ Indian Soc. Parasitol. 35, 116–122. https://doi.org/10.1007/s12639-011-0048-1

Khosravi, S., Hejazi, S.H., Hashemzadeh, M., Eslami, G., Darani, H.Y., 2012. Molecular diagnosis of Old World leishmaniasis: real-time PCR based on tryparedoxin peroxidase gene for the detection and identification of Leishmania spp. J. Vector Borne Dis. 49, 15–18.

Kuhls, K., Alam, M.Z., Cupolillo, E., Ferreira, G.E.M., Mauricio, I.L., Oddone, R., Feliciangeli, M.D., Wirth, T., Miles, M.A., Schönian, G., 2011. Comparative

microsatellite typing of new world leishmania infantum reveals low heterogeneity among populations and its recent old world origin. PLoS Negl. Trop. Dis. 5, e1155. https://doi.org/10.1371/journal.pntd.0001155

Kuhls, K., Keilonat, L., Ochsenreither, S., Schaar, M., Schweynoch, C., Presber, W., Schönian, G., 2007. Multilocus microsatellite typing (MLMT) reveals genetically isolated populations between and within the main endemic regions of visceral leishmaniasis. Microbes Infect. 9, 334–343. https://doi.org/10.1016/j.micinf.2006.12.009

Lachaud, L., Dereure, J., Chabbert, E., Reynes, J., Mauboussin, J.M., Oziol, E., Dedet, J.P., Bastien, P., 2000. Optimized PCR using patient blood samples for diagnosis and follow-up of visceral Leishmaniasis, with special reference to AIDS patients. J. Clin. Microbiol. 38, 236–240.

Lachaud, L., Marchergui-Hammami, S., Chabbert, E., Dereure, J., Dedet, J.P., Bastien, P., 2002. Comparison of Six PCR Methods Using Peripheral Blood for Detection of Canine Visceral Leishmaniasis. J. Clin. Microbiol. 40, 210–215. https://doi.org/10.1128/JCM.40.1.210-215.2002

Laskay, T., Kiessling, R., Rinke deWit, T.F., Wirth, D.F., 1991. Generation of species-specific DNA probes for Leishmania aethiopica. Mol. Biochem. Parasitol. 44, 279–286.

Laurent, T., Rijal, S., Yardley, V., Croft, S., De Doncker, S., Decuypere, S., Khanal, B., Singh, R., Schönian, G., Kuhls, K., Chappuis, F., Dujardin, J.-C., 2007. Epidemiological dynamics of antimonial resistance in Leishmania donovani: genotyping reveals a polyclonal population structure among naturally-resistant clinical isolates from Nepal. Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis. 7, 206–212. https://doi.org/10.1016/j.meegid.2006.08.005

Leite, R.S., Ferreira, S. de A., Ituassu, L.T., de Melo, M.N., de Andrade, A.S.R., 2010. PCR diagnosis of visceral leishmaniasis in asymptomatic dogs using conjunctival swab samples. Vet. Parasitol. 170, 201–206. https://doi.org/10.1016/j.vetpar.2010.02.020

Lessa, M.M., Lessa, H.A., Castro, T.W.N., Oliveira, A., Scherifer, A., Machado, P., Carvalho, E.M., 2007. Mucosal leishmaniasis: epidemiological and clinical aspects. Braz. J. Otorhinolaryngol. 73, 843–847.

Llanos-Cuentas, A., Tulliano, G., Araujo-Castillo, R., Miranda-Verastegui, C., Santamaria-Castrellon, G., Ramirez, L., Lazo, M., De Doncker, S., Boelaert, M., Robays, J., Dujardin, J.-C., Arevalo, J., Chappuis, F., 2008. Clinical and parasite species risk factors for pentavalent antimonial treatment failure in cutaneous leishmaniasis in Peru. Clin. Infect. Dis. Off. Publ. Infect. Dis. Soc. Am. 46, 223–231. https://doi.org/10.1086/524042

Lobsiger, L., Müller, N., Schweizer, T., Frey, C.F., Wiederkehr, D., Zumkehr, B., Gottstein, B., 2010. An autochthonous case of cutaneous bovine leishmaniasis in Switzerland. Vet. Parasitol. 169, 408–414. https://doi.org/10.1016/j.vetpar.2010.01.022

Lombardo, G., Pennisi, M.G., Lupo, T., Migliazzo, A., Caprì, A., Solano-Gallego, L., 2012. Detection of Leishmania infantum DNA by real-time PCR in canine oral and conjunctival swabs and comparison with other diagnostic techniques. Vet. Parasitol. 184, 10–17. https://doi.org/10.1016/j.vetpar.2011.08.010

Lukes, J., Mauricio, I.L., Schönian, G., Dujardin, J.-C., Soteriadou, K., Dedet, J.-P., Kuhls, K., Tintaya, K.W.Q., Jirků, M., Chocholová, E., Haralambous, C., Pratlong, F., Oborník, M., Horák, A., Ayala, F.J., Miles, M.A., 2007. Evolutionary and geographical history of the Leishmania donovani complex with a revision of current taxonomy. Proc. Natl. Acad. Sci. U. S. A. 104, 9375–9380. https://doi.org/10.1073/pnas.0703678104

Mahmoudzadeh-Niknam, H., Ajdary, S., Riazi-Rad, F., Mirzadegan, E., Rezaeian, A., Khaze, V., Djadid, N.D., Alimohammadian, M.H., 2012. Molecular epidemiology of cutaneous leishmaniasis and heterogeneity of *Leishmania major* strains in Iran: Molecular epidemiology of cutaneous leishmaniasis. Trop. Med. Int. Health 17, 1335–1344. https://doi.org/10.1111/j.1365-3156.2012.03078.x

Maia, C., Campino, L., 2008. Methods for diagnosis of canine leishmaniasis and immune response to infection. Vet. Parasitol. 158, 274–287. https://doi.org/10.1016/j.vetpar.2008.07.028

Maia, C., Ramada, J., Cristóvão, J.M., Gonçalves, L., Campino, L., 2009. Diagnosis of canine leishmaniasis: conventional and molecular techniques using different tissues. Vet. J. Lond. Engl. 1997 179, 142–144. https://doi.org/10.1016/j.tvjl.2007.08.009

Manna, L., Reale, S., Vitale, F., Picillo, E., Pavone, L.M., Gravino, A.E., 2008. Real-time PCR assay in Leishmania-infected dogs treated with meglumine antimoniate and allopurinol. Vet. J. Lond. Engl. 1997 177, 279–282. https://doi.org/10.1016/j.tvjl.2007.04.013

Marfurt, J., Nasereddin, A., Niederwieser, I., Jaffe, C.L., Beck, H.-P., Felger, I., 2003. Identification and Differentiation of Leishmania Species in Clinical Samples by PCR Amplification of the Miniexon Sequence and Subsequent Restriction Fragment Length Polymorphism Analysis. J. Clin. Microbiol. 41, 3147–3153. https://doi.org/10.1128/JCM.41.7.3147-3153.2003

Marfurt, Jutta, Niederwieser, I., Makia, N.D., Beck, H.-P., Felger, I., 2003. Diagnostic genotyping of Old and New World Leishmania species by PCR-RFLP. Diagn. Microbiol. Infect. Dis. 46, 115–124. https://doi.org/10.1016/S0732-8893(03)00040-3

Martinez, E., Alonso, V., Quispe, A., Thomas, M.C., Alonso, R., Piero, J.E., Gonzlez, A.C., Ortega, A., Valladares, B., 2003. RAPD method useful for distinguishing Leishmania species: design of specific primers for L. braziliensis. Parasitology 127, 513–517. https://doi.org/10.1017/S0031182003004104

Martin-Sanchez, J., Lopez-Lopez, M.C., Acedo-Sanchez, C., Castro-Fajardo, J.J., Pineda, J.A., Morillas-Marquez, F., 2001. Diagnosis of infections with Leishmania infantum using PCR-ELISA. Parasitology 122, 607–615.

Martín-Sánchez, J., Pineda, J.A., Andreu-Lopez, M., Delgado, J., Macías, J., De La Rosa, R., Morillas-Márquez, F., 2002. The high sensitivity of a PCR-ELISA in the diagnosis of cutaneous and visceral leishmaniasis caused by Leishmania infantum. Ann. Trop. Med. Parasitol. 96, 669–677. https://doi.org/10.1179/000349802125001906

Martín-Sánchez, J., Pineda, J.A., Morillas-Márquez, F., García-García, J.A., Acedo, C., Macías, J., 2004. Detection of Leishmania infantum kinetoplast DNA in peripheral blood from asymptomatic individuals at risk for parenterally transmitted infections: relationship between polymerase chain reaction results and other Leishmania infection markers. Am. J. Trop. Med. Hyg. 70, 545–548.

Mary, C., Faraut, F., Lascombe, L., Dumon, H., 2004. Quantification of Leishmania infantum DNA by a Real-Time PCR Assay with High Sensitivity. J. Clin. Microbiol. 42, 5249–5255. https://doi.org/10.1128/JCM.42.11.5249-5255.2004

Mathis, A., Deplazes, P., 1995. PCR and in vitro cultivation for detection of Leishmania spp. in diagnostic samples from humans and dogs. J. Clin. Microbiol. 33, 1145–1149.

Mauricio, I.L., Gaunt, M.W., Stothard, J.R., Miles, M.A., 2001. Genetic typing and phylogeny of the Leishmania donovani complex by restriction analysis of PCR amplified gp63 intergenic regions. Parasitology 122, 393–403.

Mauricio, I.L., Howard, M.K., Stothard, J.R., Miles, M.A., 1999. Genomic diversity in the Leishmania donovani complex. Parasitology 119 ( Pt 3), 237–246.

Mauricio, I.L., Stothard, J.R., Miles, M.A., 2004. Leishmania donovani complex: genotyping with the ribosomal internal transcribed spacer and the mini-exon. Parasitology 128, 263–267.

Maurício, I.L., Stothard, J.R., Miles, M.A., 2000. The strange case of Leishmania chagasi. Parasitol. Today Pers. Ed 16, 188–189.

Mauricio, I.L., Yeo, M., Baghaei, M., Doto, D., Pratlong, F., Zemanova, E., Dedet, J.-P., Lukes, J., Miles, M.A., 2006. Towards multilocus sequence typing of the Leishmania donovani complex: resolving genotypes and haplotypes for five polymorphic metabolic enzymes (ASAT, GPI, NH1, NH2, PGD). Int. J. Parasitol. 36, 757–769. https://doi.org/10.1016/j.ijpara.2006.03.006

Miles, M.A., Llewellyn, M.S., Lewis, M.D., Yeo, M., Baleela, R., Fitzpatrick, S., Gaunt, M.W., Mauricio, I.L., 2009. The molecular epidemiology and phylogeography of Trypanosoma cruzi and parallel research on Leishmania: looking back and to the future. Parasitology 136, 1509–1528. https://doi.org/10.1017/S0031182009990977

Minodier, P., Piarroux, R., Gambarelli, F., Joblet, C., Dumon, H., 1997. Rapid identification of causative species in patients with Old World leishmaniasis. J. Clin. Microbiol. 35, 2551–2555.

Montalvo, A.M., Fraga, J., Maes, I., Dujardin, J.-C., Van der Auwera, G., 2011. Three new sensitive and specific heat-shock protein 70 PCRs for global Leishmania species

identification. Eur. J. Clin. Microbiol. Infect. Dis. 31, 1453–1461. https://doi.org/10.1007/s10096-011-1463-z

Montalvo, A.M., Fraga, J., Monzote, L., Montano, I., De Doncker, S., Dujardin, J.C., Van Der Auwera, G., 2010. Heat-shock protein 70 PCR-RFLP: a universal simple tool for Leishmania species discrimination in the New and Old World. Parasitology 137, 1159–1168. https://doi.org/10.1017/S0031182010000089

Morales, M.A., Chicharro, C., Ares, M., Cañavate, C., Barker, D.C., Alvar, J., 2001. Molecular tracking of infections by Leishmania infantum. Trans. R. Soc. Trop. Med. Hyg. 95, 104–107.

Morales, M.A., Cruz, I., Rubio, J.M., Chicharro, C., Cañavate, C., Laguna, F., Alvar, J., 2002. Relapses versus reinfections in patients coinfected with Leishmania infantum and human immunodeficiency virus type 1. J. Infect. Dis. 185, 1533–1537. https://doi.org/10.1086/340219

Mori, Y., Notomi, T., 2009. Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. J. Infect. Chemother. Off. J. Jpn. Soc. Chemother. 15, 62–69. https://doi.org/10.1007/s10156-009-0669-9

Mugasa, C.M., Deborggraeve, S., Schoone, G.J., Laurent, T., Leeflang, M.M., Ekangu, R.A., El Safi, S., Saad, A.A., Basiye, F.L., De Doncker, S., Lubega, G.W., Kager, P.A., Büscher, P., Schallig, H.D.F.H., 2010a. Accordance and concordance of PCR and NASBA followed by oligochromatography for the molecular diagnosis of Trypanosoma brucei and Leishmania. Trop. Med. Int. Health TM IH 15, 800–805. https://doi.org/10.1111/j.1365-3156.2010.02547.x

Mugasa, C.M., Laurent, T., Schoone, G.J., Basiye, F.L., Saad, A.A., El Safi, S., Kager, P.A., Schallig, H.D., 2010b. Simplified molecular detection of Leishmania parasites in various clinical samples from patients with leishmaniasis. Parasit. Vectors 3, 13. https://doi.org/10.1186/1756-3305-3-13

Müller, N., Welle, M., Lobsiger, L., Stoffel, M.H., Boghenbor, K.K., Hilbe, M., Gottstein, B., Frey, C.F., Geyer, C., von Bomhard, W., 2009. Occurrence of Leishmania sp. in cutaneous lesions of horses in Central Europe. Vet. Parasitol. 166, 346–351. https://doi.org/10.1016/j.vetpar.2009.09.001

Mundodi, V., Somanna, A., Farrell, P.J., Gedamu, L., 2002. Genomic organization and functional expression of differentially regulated cysteine protease genes of Leishmania donovani complex. Gene 282, 257–265.

Nagamine, K., Hase, T., Notomi, T., 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol. Cell. Probes 16, 223–229.

Nasereddin, A., Bensoussan-Hermano, E., Schönian, G., Baneth, G., Jaffe, C.L., 2008. Molecular diagnosis of Old World cutaneous leishmaniasis and species identification by use of a reverse line blot hybridization assay. J. Clin. Microbiol. 46, 2848–2855. https://doi.org/10.1128/JCM.00951-08

Nicolas, L., Milon, G., Prina, E., 2002. Rapid differentiation of Old World Leishmania species by LightCycler polymerase chain reaction and melting curve analysis. J. Microbiol. Methods 51, 295–299.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28, E63.

Noyes, H., Pratlong, F., Chance, M., Ellis, J., Lanotte, G., Dedet, J.P., 2002. A previously unclassified trypanosomatid responsible for human cutaneous lesions in Martinique (French West Indies) is the most divergent member of the genus Leishmania ss. Parasitology 124, 17–24.

Noyes, H.A., Reyburn, H., Bailey, J.W., Smith, D., 1998. A nested-PCR-based schizodeme method for identifying Leishmania kinetoplast minicircle classes directly from clinical samples and its application to the study of the epidemiology of Leishmania tropica in Pakistan. J. Clin. Microbiol. 36, 2877–2881.

Ochsenreither, S., Kuhls, K., Schaar, M., Presber, W., Schönian, G., 2006. Multilocus microsatellite typing as a new tool for discrimination of Leishmania infantum MON-1 strains. J. Clin. Microbiol. 44, 495–503. https://doi.org/10.1128/JCM.44.2.495-503.2006

Oddone, R., Schweynoch, C., Schönian, G., de Sousa, C. dos S., Cupolillo, E., Espinosa, D., Arevalo, J., Noyes, H., Mauricio, I., Kuhls, K., 2009. Development of a multilocus microsatellite typing approach for discriminating strains of Leishmania (Viannia) species. J. Clin. Microbiol. 47, 2818–2825. https://doi.org/10.1128/JCM.00645-09

Oshaghi, M.A., Ravasan, N.M., Hide, M., Javadian, E.-A., Rassi, Y., Sedaghat, M.M., Mohebali, M., Hajjaran, H., 2009. Development of species-specific PCR and PCR-restriction fragment length polymorphism assays for L.infantum/L.donovani discrimination. Exp. Parasitol. 122, 61–65. https://doi.org/10.1016/j.exppara.2009.01.015

Osman, O.F., Oskam, L., Kroon, N.C., Schoone, G.J., Khalil, E.T., El-Hassan, A.M., Zijlstra, E.E., Kager, P.A., 1998a. Use of PCR for diagnosis of post-kala-azar dermal leishmaniasis. J. Clin. Microbiol. 36, 1621–1624.

Osman, O.F., Oskam, L., Zijlstra, E.E., el-Hassan, A.M., el-Naeim, D.A., Kager, P.A., 1998b. Use of the polymerase chain reaction to assess the success of visceral leishmaniasis treatment. Trans. R. Soc. Trop. Med. Hyg. 92, 397–400.

Paiva-Cavalcanti, M., Regis-da-Silva, C., Gomes, Y., 2010. Comparison of real-time PCR and conventional PCR for detection of Leishmania (Leishmania) infantum infection: a mini-review. J. Venom. Anim. Toxins Trop. Dis. 16, 537–542. https://doi.org/10.1590/S1678-91992010000400004

Pal, S., Aggarwal, G., Haldar, A., Majumdar, A., Majumdar, H.K., Duttagupta, S., 2004. Diagnosis of symptomatic kala-azar by polymerase chain reaction using patient's blood. Med. Sci. Monit. Int. Med. J. Exp. Clin. Res. 10, MT1-5.

Piarroux, R., Azaiez, R., Lossi, A.M., Reynier, P., Muscatelli, F., Gambarelli, F., Fontes, M., Dumon, H., Quilici, M., 1993. Isolation and characterization of a repetitive DNA sequence from Leishmania infantum: development of a visceral leishmaniasis polymerase chain reaction. Am. J. Trop. Med. Hyg. 49, 364–369.

Quispe Tintaya, K.W., Ying, X., Dedet, J.-P., Rijal, S., De Bolle, X., Dujardin, J.-C., 2004. Antigen genes for molecular epidemiology of leishmaniasis: polymorphism of cysteine proteinase B and surface metalloprotease glycoprotein 63 in the Leishmania donovani complex. J. Infect. Dis. 189, 1035–1043. https://doi.org/10.1086/382049

Quispe-Tintaya, K.W., Laurent, T., Decuypere, S., Hide, M., Bañuls, A.-L., De Doncker, S., Rijal, S., Cañavate, C., Campino, L., Dujardin, J.-C., 2005. Fluorogenic assay for molecular typing of the Leishmania donovani complex: taxonomic and clinical applications. J. Infect. Dis. 192, 685–692. https://doi.org/10.1086/432077

Reithinger, R., Dujardin, J.-C., 2006. Molecular Diagnosis of Leishmaniasis: Current Status and Future Applications. J. Clin. Microbiol. 45, 21–25. https://doi.org/10.1128/JCM.02029-06

Rioux, J.A., Lanotte, G., Serres, E., Pratlong, F., Bastien, P., Perieres, J., 1990a. Taxonomy of Leishmania. Use of isoenzymes. Suggestions for a new classification. Ann. Parasitol. Hum. Comparée 65, 111–125.

Rioux, J.A., Lanotte, G., Serres, E., Pratlong, F., Bastien, P., Perieres, J., 1990b. Taxonomy of Leishmania. Use of isoenzymes. Suggestions for a new classification. Ann. Parasitol. Hum. Comparée 65, 111–125.

Rocha, M.N., Margonari, C., Presot, I.M., Soares, R.P., 2010. Evaluation of 4 polymerase chain reaction protocols for cultured Leishmania spp. typing. Diagn. Microbiol. Infect. Dis. 68, 401–409. https://doi.org/10.1016/j.diagmicrobio.2010.08.007

Rodriguez, N., De Lima, H., Rodriguez, A., Brewster, S., Barker, D.C., 1997. Genomic DNA repeat from Leishmania (Viannia) braziliensis (Venezuelan strain) containing simple repeats and microsatellites. Parasitology 115 ( Pt 4), 349–358.

Rodríguez, N., Guzman, B., Rodas, A., Takiff, H., Bloom, B.R., Convit, J., 1994. Diagnosis of cutaneous leishmaniasis and species discrimination of parasites by PCR and hybridization. J. Clin. Microbiol. 32, 2246–2252.

Rodriguez, N., Rodriguez, A., Cardona, M., Barrios, M.A., McCann, S.H., Barker, D.C., 2000. Leishmania (Viannia) guyanensis: a new minicircle class exclusive to this specie isolated from a DNA cosmid library useful for taxonomic purposes. Exp. Parasitol. 94, 143–149. https://doi.org/10.1006/expr.1999.4482

Roelfsema, J.H., Nozari, N., Herremans, T., Kortbeek, L.M., Pinelli, E., 2011. Evaluation and improvement of two PCR targets in molecular typing of clinical samples of Leishmania patients. Exp. Parasitol. 127, 36–41. https://doi.org/10.1016/j.exppara.2010.06.024

Rose, K., Curtis, J., Baldwin, T., Mathis, A., Kumar, B., Sakthianandeswaren, A., Spurck, T., Low Choy, J., Handman, E., 2004. Cutaneous leishmaniasis in red kangaroos: isolation and characterisation of the causative organisms. Int. J. Parasitol. 34, 655–664. https://doi.org/10.1016/j.ijpara.2004.03.001

Rotureau, B., Ravel, C., Couppie, P., Pratlong, F., Nacher, M., Dedet, J.-P., Carme, B., 2006. Use of PCR-Restriction Fragment Length Polymorphism Analysis To

Identify the Main New World Leishmania Species and Analyze Their Taxonomic Properties and Polymorphism by Application of the Assay to Clinical Samples. J. Clin. Microbiol. 44, 459–467. https://doi.org/10.1128/JCM.44.2.459-467.2006

Saad, A.A., Ahmed, N.G., Osman, O.S., Al-Basheer, A.A., Hamad, A., Deborggraeve, S., Büscher, P., Schoone, G.J., Schallig, H.D., Laurent, T., Haleem, A., Osman, O.F., Eltom, A.M., Elbashir, M.I., El-Safi, S., 2010. Diagnostic Accuracy of the Leishmania OligoC-TesT and NASBA-Oligochromatography for Diagnosis of Leishmaniasis in Sudan. PLoS Negl. Trop. Dis. 4, e776. https://doi.org/10.1371/journal.pntd.0000776

Salotra, P., Sreenivas, G., Pogue, G.P., Lee, N., Nakhasi, H.L., Ramesh, V., Negi, N.S., 2001. Development of a species-specific PCR assay for detection of Leishmania donovani in clinical samples from patients with kala-azar and post-kala-azar dermal leishmaniasis. J. Clin. Microbiol. 39, 849–854. https://doi.org/10.1128/JCM.39.3.849-854.2001

Schallig, H.D.F.H., Oskam, L., 2002. Molecular biological applications in the diagnosis and control of leishmaniasis and parasite identification. Trop. Med. Int. Health TM IH 7, 641–651.

Schönian, G., Cupolillo, E., Mauricio, I., 2013. Molecular Evolution and Phylogeny of Leishmania, in: Ponte-Sucre, A., Diaz, E., Padrón-Nieves, M. (Eds.), Drug Resistance in Leishmania Parasites. Springer Vienna, Vienna, pp. 15–44.

SchöNian, G., Kuhls, K., Mauricio, I.L., 2010. Molecular approaches for a better understanding of the epidemiology and population genetics of Leishmania. Parasitology 138, 405–425. https://doi.org/10.1017/S0031182010001538

Schönian, G., Mauricio, I., Gramiccia, M., Cañavate, C., Boelaert, M., Dujardin, J.-C., 2008. Leishmaniases in the Mediterranean in the era of molecular epidemiology. Trends Parasitol. 24, 135–142. https://doi.org/10.1016/j.pt.2007.12.006

Schönian, G., Nasereddin, A., Dinse, N., Schweynoch, C., Schallig, H.D.F.., Presber, W., Jaffe, C.L., 2003a. PCR diagnosis and characterization of Leishmania in local and imported clinical samples. Diagn. Microbiol. Infect. Dis. 47, 349–358. https://doi.org/10.1016/S0732-8893(03)00093-2

Schönian, G., Nasereddin, A., Dinse, N., Schweynoch, C., Schallig, H.D.F.., Presber, W., Jaffe, C.L., 2003b. PCR diagnosis and characterization of Leishmania in local and imported clinical samples. Diagn. Microbiol. Infect. Dis. 47, 349–358. https://doi.org/10.1016/S0732-8893(03)00093-2

Schönian, G., Schnur, L., el Fari, M., Oskam, L., Kolesnikov, A.A., Sokolowska-Köhler, W., Presber, W., 2001. Genetic heterogeneity in the species Leishmania tropica revealed by different PCR-based methods. Trans. R. Soc. Trop. Med. Hyg. 95, 217–224.

Schubach, A., Marzochi, M.C., Cuzzi-Maya, T., Oliveira, A.V., Araujo, M.L., Oliveira, A.L., Pacheco, R.S., Momen, H., Conceicao-Silva, F., Coutinho, S.G., Marzochi, K.B., 1998. Cutaneous scars in American tegumentary leishmaniasis patients: a site of Leishmania (Viannia) braziliensis persistence and viability eleven years after antimonial therapy and clinical cure. Am. J. Trop. Med. Hyg. 58, 824–827.

Schulz, A., Mellenthin, K., Schonian, G., Fleischer, B., Drosten, C., 2003. Detection, Differentiation, and Quantitation of Pathogenic Leishmania Organisms by a Fluorescence Resonance Energy Transfer-Based Real-Time PCR Assay. J. Clin. Microbiol. 41, 1529–1535. https://doi.org/10.1128/JCM.41.4.1529-1535.2003

Schwenkenbecher, J.M., Wirth, T., Schnur, L.F., Jaffe, C.L., Schallig, H., Al-Jawabreh, A., Hamarsheh, O., Azmi, K., Pratlong, F., Schönian, G., 2006. Microsatellite analysis reveals genetic structure of Leishmania tropica. Int. J. Parasitol. 36, 237–246. https://doi.org/10.1016/j.ijpara.2005.09.010

Segatto, M., Ribeiro, L.S., Costa, D.L., Costa, C.H.N., Oliveira, M.R. de, Carvalho, S.F.G., Macedo, A.M., Valadares, H.M.S., Dietze, R., Brito, C.F.A. de, Lemos, E.M., 2012. Genetic diversity of Leishmania infantum field populations from Brazil. Mem. Inst. Oswaldo Cruz 107, 39–47.

Serin, M.S., Waki, K., Chang, K.-P., Aslan, G., Direkel, S., Otag, F., Kayar, B., Koksal, F., Emekdas, G., 2007. Consistence of miniexon polymerase chain reaction–restriction fragment length polymorphism and single-copy gene sequence analyses in discriminating Leishmania genotypes. Diagn. Microbiol. Infect. Dis. 57, 295–299. https://doi.org/10.1016/j.diagmicrobio.2006.09.001

Silveira, F.T., Ishikawa, E.A.Y., De Souza, A.A.A., Lainson, R., 2002. An outbreak of cutaneous leishmaniasis among soldiers in Belém, Pará State, Brazil, caused by

Leishmania (Viannia) lindenbergi n. sp. A new leishmanial parasite of man in the Amazon region. Parasite Paris Fr. 9, 43–50.

Simpson, A.G.B., Stevens, J.R., Lukeš, J., 2006. The evolution and diversity of kinetoplastid flagellates. Trends Parasitol. 22, 168–174. https://doi.org/10.1016/j.pt.2006.02.006

Singh, S., 2006. New developments in diagnosis of leishmaniasis. Indian J. Med. Res. 123, 311–330.

Smith, D.F., Searle, S., Ready, P.D., Gramiccia, M., Ben-Ismail, R., 1989. A kinetoplast DNA probe diagnostic for Leishmania major: sequence homologies between regions of Leishmania minicircles. Mol. Biochem. Parasitol. 37, 213–223. https://doi.org/10.1016/0166-6851(89)90153-9

Solano-Gallego, L., Koutinas, A., Miró, G., Cardoso, L., Pennisi, M.G., Ferrer, L., Bourdeau, P., Oliva, G., Baneth, G., 2009. Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis. Vet. Parasitol. 165, 1–18. https://doi.org/10.1016/j.vetpar.2009.05.022

Spanakos, G., Piperaki, E.-T., Menounos, P.G., Tegos, N., Flemetakis, A., Vakalis, N.C., 2008. Detection and species identification of Old World Leishmania in clinical samples using a PCR-based method. Trans. R. Soc. Trop. Med. Hyg. 102, 46–53. https://doi.org/10.1016/j.trstmh.2007.05.019

Strauss-Ayali, D., Jaffe, C.L., Burshtain, O., Gonen, L., Baneth, G., 2004. Polymerase chain reaction using noninvasively obtained samples, for the detection of Leishmania infantum DNA in dogs. J. Infect. Dis. 189, 1729–1733. https://doi.org/10.1086/383281

Suankratay, C., Suwanpimolkul, G., Wilde, H., Siriyasatien, P., 2010. Autochthonous visceral leishmaniasis in a human immunodeficiency virus (HIV)-infected patient: the first in thailand and review of the literature. Am. J. Trop. Med. Hyg. 82, 4–8. https://doi.org/10.4269/ajtmh.2010.09-0434

Sukmee, T., Siripattanapipong, S., Mungthin, M., Worapong, J., Rangsin, R., Samung, Y., Kongkaew, W., Bumrungsana, K., Chanachai, K., Apiwathanasorn, C., Rujirojindakul, P., Wattanasri, S., Ungchusak, K., Leelayoova, S., 2008. A suspected new species of Leishmania, the causative agent of visceral

leishmaniasis in a Thai patient. Int. J. Parasitol. 38, 617–622. https://doi.org/10.1016/j.ijpara.2007.12.003

Takagi, H., Itoh, M., Islam, M.Z., Razzaque, A., Ekram, A.R.M.S., Hashighuchi, Y., Noiri, E., Kimura, E., 2009. Sensitive, specific, and rapid detection of Leishmania donovani DNA by loop-mediated isothermal amplification. Am. J. Trop. Med. Hyg. 81, 578–582. https://doi.org/10.4269/ajtmh.2009.09-0145

Talmi-Frank, D., Nasereddin, A., Schnur, L.F., Schönian, G., Töz, S.O., Jaffe, C.L., Baneth, G., 2010. Detection and identification of old world Leishmania by high resolution melt analysis. PLoS Negl. Trop. Dis. 4, e581. https://doi.org/10.1371/journal.pntd.0000581

Thomaz-Soccol, V., Velez, I.D., Pratlong, F., Agudelos, S., Lanotte, G., Rioux, J.A., 2000. Enzymatic polymorphism and phylogenetic relationships in Leishmania Ross, 1903 (Sarcomastigophora: Kinetoplastida): a case study in Colombia. Syst. Parasitol. 46, 59–68.

Tomas-Perez, M., Fisa, R., Riera, C., 2013. The Use of Fluorescent Fragment Length Analysis (PCR-FFL) in the Direct Diagnosis and Identification of Cutaneous Leishmania Species. Am. J. Trop. Med. Hyg. 88, 586–591. https://doi.org/10.4269/ajtmh.12-0402

Tsukayama, P., Lucas, C., Bacon, D.J., 2009. Typing of four genetic loci discriminates among closely related species of New World Leishmania. Int. J. Parasitol. 39, 355–362. https://doi.org/10.1016/j.ijpara.2008.08.004

Tsukayama, P., Núñez, J.H., De Los Santos, M., Soberón, V., Lucas, C.M., Matlashewski, G., Llanos-Cuentas, A., Ore, M., Baldeviano, G.C., Edgel, K.A., Lescano, A.G., Graf, P.C.F., Bacon, D.J., 2013. A FRET-based real-time PCR assay to identify the main causal agents of New World tegumentary leishmaniasis. PLoS Negl. Trop. Dis. 7, e1956. https://doi.org/10.1371/journal.pntd.0001956

van der Meide, W., Guerra, J., Schoone, G., Farenhorst, M., Coelho, L., Faber, W., Peekel, I., Schallig, H., 2008. Comparison between quantitative nucleic acid sequence-based amplification, real-time reverse transcriptase PCR, and real-time PCR for quantification of Leishmania parasites. J. Clin. Microbiol. 46, 73–78. https://doi.org/10.1128/JCM.01416-07

van der Meide, W.F., Schoone, G.J., Faber, W.R., Zeegelaar, J.E., de Vries, H.J.C., Ozbel, Y., Lai A Fat, R.F.M., Coelho, L.I.A.R.C., Kassi, M., Schallig, H.D.F.H., 2005. Quantitative nucleic acid sequence-based assay as a new molecular tool for detection and quantification of Leishmania parasites in skin biopsy samples. J. Clin. Microbiol. 43, 5560–5566. https://doi.org/10.1128/JCM.43.11.5560-5566.2005

van Eys, G.J., Guizani, I., Ligthart, G.S., Dellagi, K., 1991. A nuclear DNA probe for the identification of strains within the Leishmania donovani complex. Exp. Parasitol. 72, 459–463.

van Eys, G.J., Schoone, G.J., Kroon, N.C., Ebeling, S.B., 1992. Sequence analysis of small subunit ribosomal RNA genes and its use for detection and identification of Leishmania parasites. Mol. Biochem. Parasitol. 51, 133–142.

van Eys, G.J., Schoone, G.J., Ligthart, G.S., Alvar, J., Evans, D.A., Terpstra, W.J., 1989. Identification of "Old World" Leishmania by DNA recombinant probes. Mol. Biochem. Parasitol. 34, 53–62.

Verma, S., Avishek, K., Sharma, V., Negi, N.S., Ramesh, V., Salotra, P., 2013. Application of loop-mediated isothermal amplification assay for the sensitive and rapid diagnosis of visceral leishmaniasis and post-kala-azar dermal leishmaniasis. Diagn. Microbiol. Infect. Dis. 75, 390–395. https://doi.org/10.1016/j.diagmicrobio.2013.01.011

Victoir, K., Bañuls, A.L., Arevalo, J., Llanos-Cuentas, A., Hamers, R., Noël, S., De Doncker, S., Le Ray, D., Tibayrenc, M., Dujardin, J.C., 1998. The gp63 gene locus, a target for genetic characterization of Leishmania belonging to subgenus Viannia. Parasitology 117 ( Pt 1), 1–13.

Villinski, J.T., Klena, J.D., Abbassy, M., Hoel, D.F., Puplampu, N., Mechta, S., Boakye, D., Raczniak, G., 2008. Evidence for a new species of Leishmania associated with a focal disease outbreak in Ghana. Diagn. Microbiol. Infect. Dis. 60, 323–327. https://doi.org/10.1016/j.diagmicrobio.2007.09.013

Vitale, F., Reale, S., Vitale, M., Petrotta, E., Torina, A., Caracappa, S., 2004. TaqMan-based detection of Leishmania infantum DNA using canine samples. Ann. N. Y. Acad. Sci. 1026, 139–143. https://doi.org/10.1196/annals.1307.018

Waki, K., Dutta, S., Ray, D., Kolli, B.K., Akman, L., Kawazu, S.-I., Lin, C.-P., Chang, K.-P., 2007. Transmembrane molecules for phylogenetic analyses of pathogenic protists: Leishmania-specific informative sites in hydrophilic loops of trans-endoplasmic reticulum N-acetylglucosamine-1-phosphate transferase. Eukaryot. Cell 6, 198–210. https://doi.org/10.1128/EC.00282-06

Weirather, J.L., Jeronimo, S.M.B., Gautam, S., Sundar, S., Kang, M., Kurtz, M.A., Haque, R., Schriefer, A., Talhari, S., Carvalho, E.M., Donelson, J.E., Wilson, M.E., 2011. Serial quantitative PCR assay for detection, species discrimination, and quantification of Leishmania spp. in human samples. J. Clin. Microbiol. 49, 3892–3904. https://doi.org/10.1128/JCM.r00764-11

Welsh, J., McClelland, M., 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 18, 7213–7218.

Williams, J.G., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V., 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18, 6531–6535.

Wortmann, G., Hochberg, L., Houng, H.-H., Sweeney, C., Zapor, M., Aronson, N., Weina, P., Ockenhouse, C.F., 2005. Rapid identification of Leishmania complexes by a real-time PCR assay. Am. J. Trop. Med. Hyg. 73, 999–1004.

Yurchenko, V.Y., Lukes, J., Jirku, M., Zeledón, R., Maslov, D.A., 2006. Leptomonas costaricensis sp. n. (Kinetoplastea: Trypanosomatidae), a member of the novel phylogenetic group of insect trypanosomatids closely related to the genus Leishmania. Parasitology 133, 537–546. https://doi.org/10.1017/S0031182006000746

Zelazny, A.M., Fedorko, D.P., Li, L., Neva, F.A., Fischer, S.H., 2005. Evaluation of 7SL RNA gene sequences for the identification of Leishmania spp. Am. J. Trop. Med. Hyg. 72, 415–420.

Zemanová, E., Jirků, M., Mauricio, I.L., Horák, A., Miles, M.A., Lukeš, J., 2007. The Leishmania donovani complex: Genotypes of five metabolic enzymes (ICD, ME, MPI, G6PDH, and FH), new targets for multilocus sequence typing. Int. J. Parasitol. 37, 149–160. https://doi.org/10.1016/j.ijpara.2006.08.008

Zemanová, E., Jirků, M., Mauricio, I.L., Miles, M.A., Lukes, J., 2004. Genetic polymorphism within the leishmania donovani complex: correlation with geographic origin. Am. J. Trop. Med. Hyg. 70, 613–617.

Zhang, C.-Y., Lu, X.-J., Du, X.-Q., Jian, J., Shu, L., Ma, Y., 2013. Phylogenetic and Evolutionary Analysis of Chinese Leishmania Isolates Based on Multilocus Sequence Typing. PLoS ONE 8, e63124. https://doi.org/10.1371/journal.pone.0063124

# CHAPTER II

## FIRST EVIDENCE OF *LEISHMANIA* INFECTION IN EUROPEAN BROWN HARE (*LEPUS EUROPAEUS*) IN GREECE, GIS ANALYSIS AND PHYLOGENETIC POSITION WITHIN THE *LEISHMANIA SPP*

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## Abstract

Although the existence of a sylvatic transmission cycle of *Leishmania* spp, independent from the domestic cycle, has been proposed, data are scarce on *Leishmania* infection in wild mammals in Greece. In this study we aimed to investigate the presence of *Leishmania* infection in the European brown hare in Greece, to infer the phylogenetic position of the *Leishmania* parasites detected in hares in Greece and to identify any possible correlation between *Leishmania* infection in hares with environmental parameters, using the geographical information system (GIS). Spleen samples from 166 hares were tested by internal transcribed spacer-1 (ITS-1) nested PCR for the detection of *Leishmania* DNA. Phylogenetic analysis was performed on *Leishmania* sequences from hares in Greece in conjunction with *Leishmania* sequences from dogs in Greece and 46 *Leishmania* sequences retrieved from GenBank. The *Leishmania* DNA prevalence in hares was found to be 23.49% (95% CI: 17.27 - 30.69). The phylogenetic analysis confirmed that the *Leishmania* sequences from hares in Greece belong in the *Leishmania donovani* complex. The widespread *Leishmania* infection in hares should be taken into consideration because under specific circumstances this species can act as a reservoir host. This study suggests that the role of wild animals, including hares, in the epidemiology of *Leishmania spp* in Greece deserves further elucidation.

## Introduction

Leishmaniasis is a vector-borne mammalian disease caused by a protozoan flagellate of the genus *Leishmania*, transmitted by phlebotomine sandfly species. Dogs are the major reservoir host of *Leishmania infantum*. However, *Leishmania* infection has been also described in cats, equines and wild mammals including lagomorphs (Gramiccia 2011; Molina et al. 2012; Ruiz-Fons et al. 2013; Moreno et al. 2014; Jiménez et al. 2014; Souza et al. 2014).

Greece is considered to be an endemic country for leishmaniasis. The visceral form of the disease, caused by *L. infantum,* is the predominant one, endemic in nearly all geographical areas of the country. Cutaneous leishmaniasis caused by *L. tropica* and *L. infantum* in some cases*,* is also present but it occurs sporadically (Gkolfinopoulou et al. 2013). The overall reported seroprevalence in the canine population in seven regions of the Greek mainland was nearly 20% ranging from 2.05% in Florina to 30.12% in Attiki (Athanasiou et al. 2012) while the seroprevalence was 3.87% in clinically healthy stray cats living in the area of Thessaloniki (Diakou et al. 2009) and a very low seroprevalence (0.3%) has been reported in horses in Attiki (Kouam et al. 2010).

Although the existence of a sylvatic transmission cycle that is independent from the domestic cycle has been proposed (Sobrino et al. 2008; Molina et al. 2012; Millán et al. 2014), there is a scarcity of data on *Leishmania* infection in wild mammals in Greece, a highly endemic country for canine leishmaniasis. Regarding the lagomorphs, there are no data in the countries of the Mediterranean basin including Greece. However, several studies conducted in Spain, revealed a 43.6% *Leishmania* DNA prevalence (Ruiz-Fons et al. 2013) and 74.1% seroprevalence in hares (Moreno et al. 2014). Moreover, four out of seven apparently healthy hares submitted to xenodiagnosis, were able to infect a mean 4.7%

*Phlebotomus perniciosus* sand flies (0–10.6%), a competent *L.infantum* vector, suggesting that hares may represent a secondary reservoir in the sylvatic transmission cycle of *L. infantum* in that region (Molina et al. 2012).

Sequence data of the ribosomal RNA (rRNA) gene, in particular the two highly variable internal transcribed spacer regions (ITS-1 and ITS-2), have been used to resolve taxonomic questions and to determine phylogenetic affinities among closely related

*Leishmania* species (Dávila et al. 2000; Schönian et al. 2000; El Tai et al. 2001; Kuhls et al. 2005; Parvizi et al. 2008; Yang et al. 2010; Wang et al. 2010; Hajjaran et al. 2013). The sequences of seven *Leishmania* parasites detected in hares were used for the phylogenetic analysis in conjunction with three sequences of *Leishmania* parasites detected in dogs in Greece and 46 *Leishmania* sequences retrieved from GenBank. Our work is a first attempt to investigate the presence of *Leishmania* infection in hares (*Lepus europaeus*) in Greece, to identify the environmental parameters related to *Leishmania* infection in hares using GIS and statistical analysis and to infer the phylogenetic position of the *Leishmania* sequences from hares in Greece.

## Materials and Methods

### Hare and canine samples, DNA extraction, ITS-1 nested PCR and Sequencing

Spleen hare samples (n=166) were collected in the prefectures of Thessaloniki (n=82) and Chalkidiki (n=84) (Northern Greece) from 2007 to 2011. The samples were stored at the Research Division, Hunting Federation of Macedonia and Thrace, Thessaloniki, Greece, and later submitted still frozen to the Laboratory of Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Thessaly, Greece, where they were stored at −80°C. Data on hare specimens were located in the field using handheld global positioning system (GPS) units. Canine lymph node aspirates were collected from three dogs with diagnosed canine leishmaniasis in the prefecture of Thessaloniki. The canine samples were submitted to the Laboratory of Microbiology and Parasitology, University of Thessaly, Greece, within 24 hours after collection and they were immediately stored at -20˚C pending DNA extraction.

Total genomic DNA extraction was performed using a commercially available DNA extraction kit (Thermo Scientific GeneJET Genomic DNA Purification Kit) according to the manufacturer's protocol. The purified DNA was stored at -20˚C.

The samples were analyzed by ITS-1 nested PCR (ITS-1 nPCR) as described previously (Leite et al. 2010). Primers addressed to ITS-1 between the genes coding for SSU rRNA and 5.8SrRNA were used. For the first amplification 5 μl of DNA solution was added to 45.0 μl of PCR mix containing 25 pmol of the primers 5'-CTGGATCATTTTCCGATG-3' and 5'-TGATACCACTTATCGCACTT-3' and 0.2mM deoxynucleoside triphosphates, 1.5 mM MgCl2, 5mM KCl, 75mM Tris–HCl pH 9.0, and 2U of AmpliTaq DNA polymerase (Applied

Biosystems). The cycling conditions were initial denaturation at 95 ◦C for 2min followed by 34 cycles consisting of denaturation at 95 ◦C for 20 s, annealing at 53 ◦C for 30 s, and extension at 72 ◦C for 1 min, followed by a final extension at 72 ◦C for 6 min. Amplification products were subjected to electrophoresis in 2% agarose gel stained with ethidium bromide (0.5 μg/ml) and visualized under ultraviolet light. The PCR product size stays between 300 and 350 bp. For the second amplification 10.0 μl of a 1:40 dilution of the first PCR product was added to 15 μl of PCR mix under the same conditions as the first amplification but with the following primers: 5'-CATTTTCCGATGATTACACC-3' and 5'-CGTTCTTCAACGAAATAGG-3'. Amplification products were visualized on 2% agarose gel stained with ethidium bromide (0.5 μg/ml). The PCR product size stays between 280 and 330bp.

Amplicons from seven randomly selected *Leishmania* PCR positive hares were sequenced in order to perform the initial identification of the phylogenetic position of the *Leishmania* parasites detected in hares within the *Leishmania* spp. Furthermore, amplicons from three *Leishmania* PCR positive dogs were sequenced. Sequence analysis was performed on a part of the rRNA gene (ITS-1 region). The positive PCR product was purified using the PureLink PCR purification kit (Invitrogen) and was bidirectionally sequenced using the fluorescent BigDye Terminator Cycle sequencing kit v3.1 (Applied Biosystems), followed by fragment separation with a 3,730xl DNA analyzer (Applied Biosystems). The sequences have been deposited in GenBank under accession numbers KP410398- KP410407 (Table 1) and they were used for the phylogenetic analysis in this study.

Table 1. List of database accession numbers and origin of the *Leishmania* strains used in this study.

| *Leishmania* species | GenBank accession number | Origin |
|---|---|---|
| *L.donovani* | AJ634360 | Ethiopia |
| *L.donovani* | AJ634359 | Sudan |
| *L.donovani* | AM901450 | India |
| *L.infantum* | GQ444144 | Iran |
| *L.infantum* | FM164420 | Uzbekistan |
| *L.infantum* | EU326227 | Brazil |
| *L.infantum* | AJ634355 | Spain |
| *L.infantum* | AJ634354 | Italy |
| *L.infantum* | AJ634351 | France |
| *L.infantum* | AJ634350 | Malta |
| *L.infantum* | AJ634344 | Portugal |
| *L.chagasi* | AJ000306 | Brazil |
| *L.infantum* | AJ000289 | Tunisia |
| *L. infantum* | GQ367487 | China |
| *L.donovani* | AJ000294 | China |
| *L.donovani* | HM130608 | China |
| *L.donovani* | AM901452 | Iraq |
| *L.donovani* | EU753232 | India |
| *L.donovani* | AM901449 | India |
| *L.donovani* | AJ276258 | Sudan |
| *L.donovani* | AM901453 | Morocco |
| *L.donovani* | AJ249614 | Sudan |

| | | |
|---|---|---|
| *L.donovani* | AJ276260 | Sudan |
| *L.donovani* | AM901448 | Sri lanka |
| *L.donovani* | AJ634374 | Kenya |
| *L.donovani* | AJ634373 | Ethiopia |
| *L.infantum*$^{\alpha}$ | AJ634371 | Sudan |
| *L.donovani* | AJ249616 | Sudan |
| *L.donovani* | AJ249615 | Sudan |
| *L.donovani* | AJ249621 | Sudan |
| *L.donovani complex* (GRE 3 hare) | KP410403 | Greece |
| *L.donovani complex* (GRE 1 dog) | KP410398 | Greece |
| *L.donovani complex* (GRE 2 dog) | KP410399 | Greece |
| *L.donovani complex* (GRE 3 dog) | KP410400 | Greece |
| *L.donovani complex* (GRE 1 hare) | KP410401 | Greece |
| *L.donovani complex* (GRE 2 hare) | KP410402 | Greece |
| *L.donovani complex* (GRE 5 hare) | KP410405 | Greece |
| *L.donovani complex* (GRE 6 hare) | KP410406 | Greece |
| *L.donovani complex* (GRE 7 hare) | KP410407 | Greece |

| | | |
|---|---|---|
| *L.donovani complex* | KP410404 | Greece |
| (GRE 4 hare) | | |
| *L.tropica* | AJ300485 | Tunisia |
| *L.tropica* | GQ913688 | Afghanistan |
| *L.tropica* | FJ460459 | India |
| *L.major* | AY260965 | Iran |
| *L.major* | AJ300482 | Kenya |
| *L.major* | AJ272383 | Turkmenistan |
| *L.turanica* | EF413079 | Iran |
| *L.turanica* | AJ272378 | Uzbekistan |
| *L.turanica* | AJ272379 | Turkmenistan |
| *L.gerbilli* | AJ300486 | Uzbekistan |
| *L.aethiopica* | GQ920677 | - |
| *L.aethiopica* | GQ920673 | - |
| *L.mexicana* | AF466383 | - |
| *L.mexicana* | FJ948433 | - |
| *L.amazonensis* | AF339753 | - |
| *L.panamensis* | FJ948442 | - |

[a]Identified as *L.infantum* according to the zymodeme, MON30, whereas recent analyses have shown that it is *L.donovani* (Zemanová et al. 2004; Jamjoom et al. 2004).

## Geographical Information System (GIS) and statistical analysis

The GIS layers were obtained from climate, elevation and land cover data bases acquired from the network. Altitude was extracted from a digital elevation model (DEM) with a spatial resolution of 1 square kilometer. Land uses were determined from the Corine Land Cover 2000 database (European Environment Agency-EEA) and the ArcGIS online application. Climate data were derived from World Clim database.

The livestock raw data were provided from Payment and Control Agency for Guidance and Guarantee Community Aid. New layers were created to represent the towns and villages, the distance from the nearest village, the distance from water presence and the density of livestock. Crop types were calculated in a buffer zone of 1km from the sample coordinates (Table 2). All data layers were converted to a common projection, map extent and resolution. ArcGIS 10.1 GIS software (ESRI, Redlands, CA, USA) was used for data analysis.

**Table 2.** Environmental variables used in the analysis

| Variable | Value | Source |
|---|---|---|
| Slope (degrees) at 500- and 1000-m radii | X, SD, min, max | DEM |
| Topographic position index (4 classes) | binary | DEM |
| Altitude | continuous | DEM |
| Aspect | N,W,S,E | DEM |
| Distance from nearest village (m) | continuous | ArcGIS-WMS |
| Distance from water (m) | continuous | ArcGIS-DEM |
| Distance from rivers (m) | continuous | ArcGIS-DEM |
| Distance from game refuges | continuous | GEoDatabase |
| Distance from road network (2 classes: forest-paved) | continuous | GEoDatabase |
| Distance from livestock farms | continuous | GEoDatabase |
| Farms density-livestock density | continuous | GEoDatabase |
| Habitat types (9 classes: Forests, cultivations, etc.) | Categorical transform to continuous | ArcGIS-Corine LC (EEA) |
| 19 Climatic variables (Temperature 11 indices, Precipitation 8 indices) | continuous | World Clim Database |
| NDVI (Normalized Difference Vegetation Index) 12 indices | continuous | World Clim Database |
| Population density | continuous | GEoDatabase |

Statistical analysis was performed with IBM SPSS 22.0 using basic descriptive and multivariate statistics (Gray and Kinnear 2012). We used the aforementioned bioclimatic and environmental variables in order to test if the bivariate dependent variable (DV) - *Leishmania* positive or negative hares - is related to some of the independent variables (IV). Because of the big set of IVs, we first tried to find some

factors (new variables) which may represent the most of the variability of the data. We used the exploratory factor analysis for this. The purpose was to use these factors in a bivariate logistic regression model that would be able to predict the positive hares when some IVs would take a range of values (Tabachnick and Fidell 2007). We set the significance level to p = 5%.

## Phylogenetic analysis

Molecular evolutionary analyses were conducted on nucleotide sequences of *Leishmania* parasites detected in hares and dogs in Greece and on *Leishmania* sequences that were retrieved from the EMBL database, using the program MEGA 6 (Table 1).

To determine the appropriate model of sequence evolution and statistically compare successively nested more parameter-rich models for this data set, the program MODELTEST Version 3.6 (Posada and Crandall 1998) was used. With a statistical significance of P=0.01 the HKY85 model (Hasegawa et al. 1985), with gamma correction, obtained the best likelihood score and was thus selected for subsequent analysis. Maximum parsimony (MP) tree was constructed under the heuristic search option with 100 random-taxon-addition replicates and tree bisection–reconnection branch swapping, using PAUP*. Node support was assessed on the basis of 1000 bootstrap replicates.

A Bayesian analysis was also performed with MRBAYES version 3.1 (Huelsenbeck and Ronquist 2001), under the HKY85 model of sequence evolution. Depending on the data set, random starting trees run for $2 \times 10^6$ to $8 \times 10^6$ generations were used, sampled every 100 generations. Burn-in frequency was set to the first 25% of the sampled trees. With some minor differences, the phylogenetic tree based on the Bayesian analysis as well as MP tree showed similar topologies (the tree is available on request).

## Results

### ITS-1 nested PCR and Sequencing

Overall 39 out of 166 hare samples (23.49%, 95% CI: 17.27 - 30.69) were positive for the presence of *Leishmania* DNA (Thessaloniki 17.1%, 95% CI: 9.7-27.0 and Chalkidiki 29.8%, 95% CI: 20.3-40.7). The distribution of the *Leishmania* positive hares is shown

in Fig. 1. The three canine samples collected from dogs diagnosed with canine leishmaniasis, were PCR positive. Seven amplicons from *Leishmania* PCR positive hares and the three amplicons from *Leishmania* PCR positive dogs were sequenced.

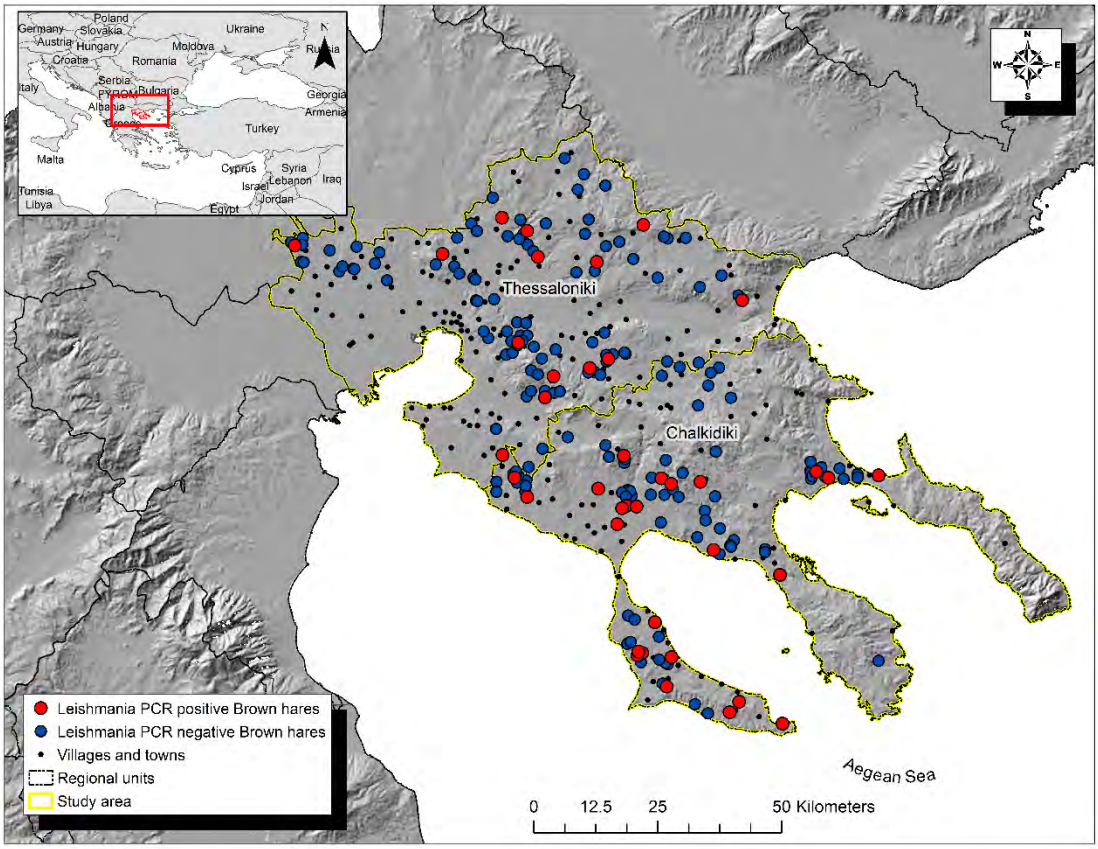


**Figure 1. Map of Greece showing the geographical distribution of *Leishmania* PCR positive hares**. Map of Greece showing the geographical distribution of *Leishmania* PCR positive hares between 2007 and 2011 in the prefectures of Thessaloniki and Chalkidiki. Red and blue dots indicate the *Leishmania* PCR positive and the *Leishmania* PCR negative hares respectively.

## GIS and statistical analysis

The *Leishmania* positive hares were found in shrubland with pastures, in agricultural areas and in broadleaved forests with a mean altitude of 225.38 m asl (range 35-750 ± 200.48 SD) while the mean distance from villages and towns was 2.472 m asl (range 850-4661± 1029.02 SD). Fifty-four percent and 42% of the positive hare samples were found in crop types of wheat fields and in olive tree plantations respectively. The remaining 4% of the positive hare samples were found in cotton and forage plants

types. The mean livestock density in the study area (1km buffer zone) was 38.42 livestock animals (range 0-200 ± 47.36 SD). The number of sheep and goats ranged from 5 to 880 (± 151.9 SD) and the number of cattle ranged from 2 to 330 (± 77.48 SD). The positive hare samples were detected within a mean distance of 1169.85 m (range 10-7285 ± 1240.94 SD) from bodies of water. The mean annual temperature was 14.8 $^{0}$C (range 11.7-16.4 ± 12.24 SD) in the prefectures studied during the study period while the mean annual precipitation recorded was 474.82 mm (range 443-527 ± 23.76 SD) and the annual average humidity reached 67.5%.

We used the exploratory factor analysis three times. One to reduce the variables related to temperature, one to reduce the variables related to precipitation and one for the vegetation index variables. Using the criterion of eigenvalues of more than 1, the procedure generated three factors for every group of variables. By entering these variables to the bivariate logistic regression procedure, together with the variables related to the distance from rivers, villages etc., we did not find a single significant relationship between the DV and the IVs. We then checked the simple biserial correlations between the DV and the IVs. We found that five bioclimatic variables were correlated with the DV, as shown in Table 3. We used these variables in a logistic regression procedure and found only one slightly significant relationship. We found that the presence of *Leishmania* DNA in hares may be influenced by the precipitation seasonality variable. This variable is produced by the division of the standard deviation of the annual precipitation with the mean value of annual precipitation, that is, the coefficient of variation. However, this relationship is significant only at the 90% level. Table 3 shows the results of the logistic regression. We can interpret this finding as follows: we are 90% sure that an increase of one unit in the precipitation seasonality is expected to increase by 6.1% the odds of hares to be *Leishmania* positive. This means that in such a case a hare will have a probability of about 52% to be *Leishmania* positive. Since we do not know the real probability for positives (instead of the initial scenario of 50% probability) the above finding may be indicative. However, the fact that the 95% confidence interval for the odds includes value 1 should be taken into consideration.

Table 3. Independent variables related to the dependent one

| | Mean Temperature of Coldest Quarter | Precipitation Seasonality (Coefficient of Variation) | Mean Diurnal Range (Mean of monthly (max temp _ min temp)) | Temperature Seasonality (standard deviation *100) | Temperature Annual Range |
|---|---|---|---|---|---|
| Biserial correlation R | 0.167 | 0.184 | -0.178 | -0.170 | 0.161 |
| p-value for R | 0.017 | 0.008 | 0.011 | 0.015 | 0.021 |
| Odds | | 1.061 | | | |
| p-value for Odds | | 0.082 | | | |
| 95% CI for odds | | 0.993 – 1.134 | | | |

## Phylogenetic analysis

The phylogenetic analysis performed on 56 *Leishmania* sequences, including seven Greek hare and three Greek canine *Leishmania* sequences revealed that the homology of the nucleotide sequences between the seven Greek *Leishmania* sequences from hares was 99.1%. The homology of the nucleotide sequences between the seven hare and the three canine *Leishmania* sequences from Greece was 98.9%, as well as the homology of the nucleotide sequences between the Greek *Leishmania* sequences and the strains belonging to *Leishmania donovani* complex which were retrieved from GenBank.

The Greek sequences belonged to the *L.donovani* complex and they did not form a monophyletic group. Two major clades (I and II) could be recognized for the *L. donovani* complex in the maximum parsimony tree based on the ITS-1 sequences (Fig. 2). Clade I included six *Leishmania* sequences detected in hares in Greece. These sequences formed a strongly supported clade (Clade I: *Leishmania sp*.; BP=81%) which was further divided into two subclades (Ia and Ib). Clade II included three canine (GRE1 dog, GRE2 dog, GRE3 dog) and one hare *Leishmania* sequence from Greece (GRE3 hare) and all

the *L. donovani* complex strains retrieved from GenBank. This clade was further divided into two subclades (IIa and IIb).

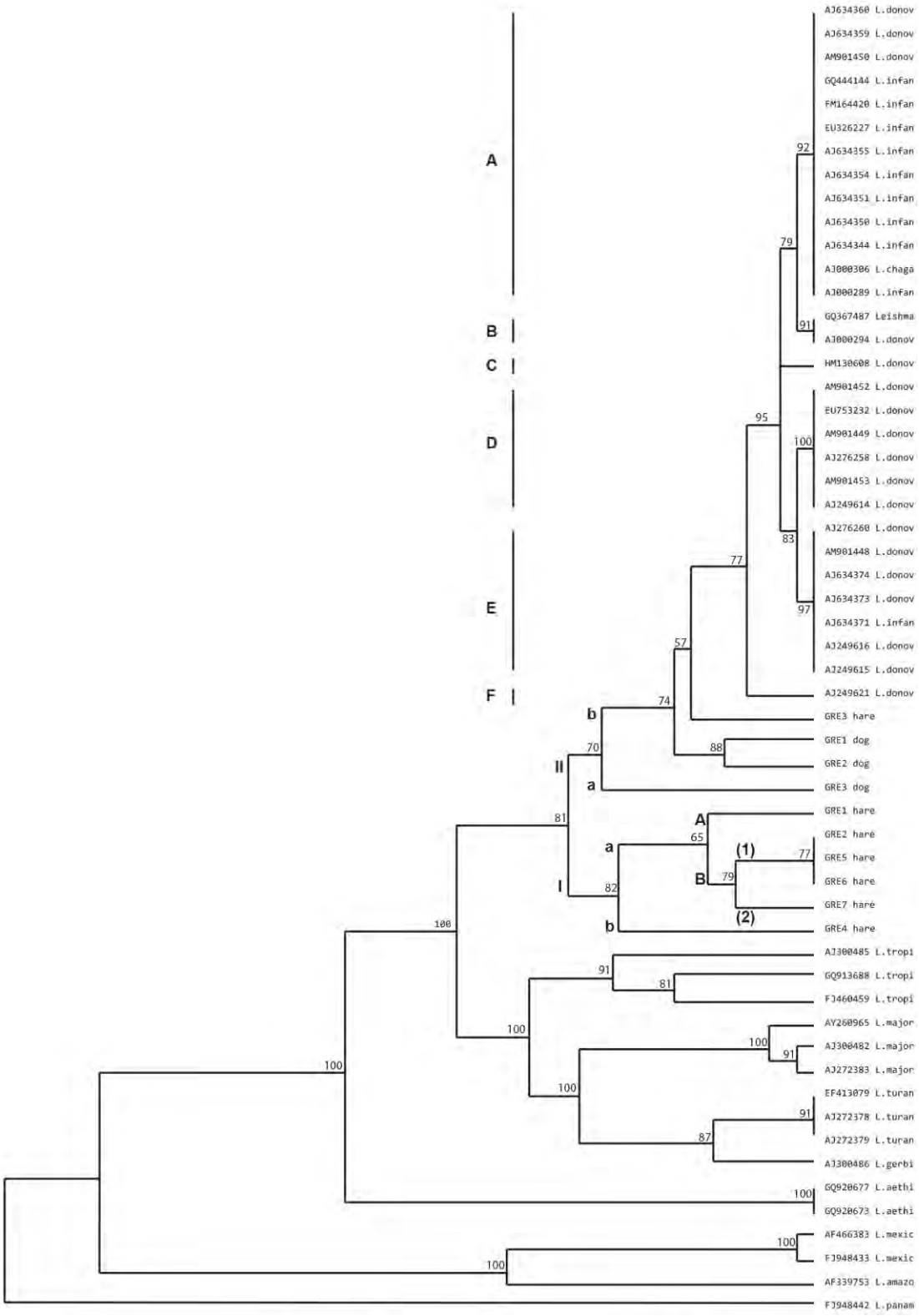

Figure 2. Phylogenetic tree constructed based on ITS-1 sequences of 56 *Leishmania* isolates in this study Phylogenetic tree based on Maximum Parsimony analysis from 56 *Leishmania* sequences (ITS-1 between the genes coding for SSU rRNA and 5.8SrRNA) including the 10 Greek *Leishmania* sequences under study, GRE1 hare, GRE2 hare, GRE3 hare, GRE4 hare, GRE5 hare, GRE6 hare, GRE7 hare, GRE1 dog, GRE2 dog, GRE3 dog. With some minor differences, the phylogenetic tree based on the Bayesian analysis (available on request) showed similar topologies. The main branches exhibited high bootstrap values and high posterior probabilities (Bayesian analysis) and clearly separated groups in distinct clades.

## Discussion

This study was a preliminary attempt to detect *Leishmania* infection in European brown hares (*Lepus europaeus*) in Greece and to identify the environmental parameters related to *Leishmania* infection using GIS and statistical analysis. Moreover, we aimed to infer the phylogenetic position of the *Leishmania* sequences from hares and dogs in Greece. This is the first report of *Leishmania* infection in hares in Greece and our results are in close agreement with the previous reports regarding the susceptibility of hares to *Leishmania* infection (Ruiz-Fons et al. 2013). The high prevalence of infection (23.49%) found in our study together with the infectiousness of hares to sand flies and the feeding preference of the latter for hares as documented earlier in Spain (Molina et al. 2012; Jiménez et al. 2013), raises a concern about the role of hares in the epidemiology of *Leishmania spp* in Greece. Moreover, the similarity detected between the hare and the canine *Leishmania* sequences from Greece (98.9%. homology of the nucleotide sequences), could probably be indicative of a possible overlapping of wild and domestic transmission cycles of *Leishmania spp.* However, this hypothesis needs further investigation.

Interestingly, the GIS analysis revealed that the positive hare samples were found in a mean altitude of 225.38 meters above sea level (range 35-750 ± 200.48 SD) while the mean distance was 2.472 m (range 850-4661± 1029.02 SD) and 1169.85 m (range 10-7285 ± 1240.94 SD) from villages and towns and bodies of water respectively. Moreover, in a buffer zone of 1 km the mean livestock density was 38.42 (range 0-200

± 47.36 SD). The *Leishmania* positive hares were found in areas with 67.5% annual average humidity, 474.82 mm (range 443-527 ± 23.76 SD) mean annual precipitation and 14.8 $^{0}$C (range 11.7-16.4 ± 12.24 SD) mean annual temperature. The statistical analysis showed that the presence of *Leishmania* DNA in hares may be influenced by the precipitation seasonality variable**,** suggesting that an increase of one unit in the precipitation seasonality is expected to increase the odds of hares to be *Leishmania* positive by 6.1%. However, this relationship was significant only at the 90% level and it should be correlated with the vector abundance and the phlebotomine sandfly species diversity.

The hare and canine *Leishmania* sequences in this study had a basal position in the *L.donovani* complex. Six hare sequences (Clade I) formed a separate branch from the three canine and one hare *Leishmania* sequence from Greece and all the *L.donovani* complex strains retrieved from GenBank (Clade II).

The mean annual incidence of reported human leishmaniasis cases in Greece between 1998 and 2011 was 0.36 cases per 100,000 population, with fluctuation during this period, generally decreasing after 2007, with a small re-increase in 2011 (Gkolfinopoulou et al. 2013). During our study time period, three human cases were reported in the prefecture of Thessaloniki whereas no human cases were reported in the prefecture of Chalkidiki (HCDCP). Regarding the canine population, the seropositivity reported from 2005-2010 was 29.81-42.00% in Thessaloniki and 0.01-10.66% in Chalkidiki (Ntais et al. 2013). Thus far, we have no indication that there is an association between the human leishmaniasis cases and the infected hares in the prefectures studied partly due to the low number of officially reported human cases and also probably because they do not share the same living space. Moreover, the *Leishmania* DNA prevalence found in hares in Chalkidiki and Thessaloniki in conjunction with the seropositivity rates in canine population (Ntais et al. 2013) and the reported human leishmaniasis cases (HCDCP) in the same prefectures during the same study period leads to the hypothesis that leishmaniasis is still in the sylvatic transmission cycle in Chalkidiki whereas it seems that an overlapping of sylvatic and domestic transmission cycles occurs in Thessaloniki. It is well known that leishmaniasis involves a complex interplay between the protozoon pathogen *Leishmania*, the arthropod vectors sandflies, the environmental influence on vector distribution, the primary and

secondary reservoirs of infection and the susceptible human populations thus making necessary the simultaneous investigation of these factors in order to infer the role of a mammalian host in the epidemiology of leishmaniasis. However, the investigation of *Leishmania* infection in different hosts is crucial in order to design further epidemiological studies, control and prevention strategies. Thus, the widespread *Leishmania* infection in hares in the prefectures studied in Greece should be taken into consideration because as reported by Molina et al. (2012), under specific circumstances including an unusual increase in hare population, high density of sandflies and a low level of immunity in the human population, this species can act as a reservoir host.

Further studies including *Leishmania* sequences from human cases, domestic and wild animals and sandflies from Greece and the neighboring countries will give insight into the phylogenetic position of the *Leishmania spp* circulating in Greece and the evolutionary history among Greek isolates. The role of the different hosts in the epidemiology of leishmaniasis in Greece deserves further investigation together with the estimation of the vector abundance, distribution and diversity. In particular, the role of wild mammals in the transmission cycle of *Leishmania spp* which remains largely unknown in Greece, a highly endemic country for canine leishmaniasis, needs further elucidation. Studies in endemic and non-endemic areas in the mainland and the islands of Greece, including the vector abundance and the species diversity, will reveal the role of hares in the epidemiology of *Leishmania spp* in Greece.

## Compliance with ethical standards

The hare samples examined for the presence of *Leishmania* DNA in this study, represent archived material collected opportunistically (no active capture, killing and sampling of wild animals specifically for this study was performed) from animals hunter-harvested by members of the Greek Hunting Federation of Macedonia and Thrace, during the hunting seasons, according to the prerequisites of the Greek Legislation (Ministerial Decision 103305/6093/14-11-2007, FEK 1626/Β΄/13.8.2008, FEK 1611/Β΄/2009, FEK 1183/6-8-2010, FEK 1763/4-8-2011), or from hares found dead and submitted to our laboratory for Passive Wildlife Disease surveillance during the period 2007–2011. Thus, special approval was not necessary and steps to ameliorate suffering were not applicable in this study. Research on animals as defined in the EU Ethics for

Researchers document (European Commission, 2007, Ethics for Researchers - Facilitating Research Excellence in FP7, Luxembourg: Office for Official Publications of the European Communities, ISBN 978-92-79-05474-7) is not a part of the study.

The canine samples included in this study were collected by private practicing veterinarians. No animals were euthanised during the study and efforts taken to ameliorate animal suffering. The study did not involve any experimentation but was based on samples that had been collected from the dogs for routine diagnostic purposes. Diagnostic veterinary procedures are not within the context of relevant EU legislation for animal experimentations (Directive 86/609/EC) and may be performed in order to diagnose animal diseases and improve animal welfare.

## References


Athanasiou LV, Kontos VI, Saridomichelakis MN, Rallis TS, Diakou A (2012) A cross-sectional sero-epidemiological study of canine leishmaniasis in Greek mainland. Acta Trop 122: 291–295.

Dávila AM, Momen H (2000) Internal-transcribed-spacer (ITS) sequences used to explore phylogenetic relationships within *Leishmania*. Ann Trop Med Parasitol 94: 651–654.

Diakou A, Papadopoulos E, Lazarides K (2009) Specific anti-*Leishmania* spp. antibodies in stray cats in Greece. J Feline Med Surg 11: 728–730.

El Tai NO, El Fari M, Mauricio I, Miles MA, Oskam L, El Safi SH, Presber WH, Schönian G (2001) *Leishmania donovani*: intraspecific polymorphisms of Sudanese isolates revealed by PCR-based analyses and DNA sequencing. Exp Parasitol 97: 35–44.

Gkolfinopoulou K, Bitsolas N, Patrinos S, Veneti L, Marka A, Dougas G, Pervanidou D, Detsis M, Triantafillou E, Georgakopoulou T, Billinis C, Kremastinou J, Hadjichristodoulou C (2013) Epidemiology of human leishmaniasis in Greece, 1981-2011. Euro Surveill 18(29):pii=20532.

Gramiccia M (2011) Recent advances in leishmaniosis in pet animals: epidemiology, diagnostics and anti-vectorial prophylaxis. Vet Parasitol 181: 23–30.

Gray CD, Kinnear PR (2012) IBM SPSS statistics 19 made simple. Psychology Press.

Hajjaran H, Mohebali M, Mamishi S, Vasigheh F, Oshaghi MA, Naddaf S R, Teimouri A, Edrissian G H, Zarei Z (2013) Molecular identification and polymorphism determination of cutaneous and visceral leishmaniasis agents isolated from human and animal hosts in Iran. BioMed Res Int: 789326.

Hasegawa M, Kishino H, Yano T (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J Mol Evol 22: 160–174.

Huelsenbeck JP, Ronquist F. (2001) MRBAYES: Bayesian inference of phylogenetic trees. Bioinforma Oxf Engl 17: 754–755.

Jamjoom MB, Ashford RW, Bates PA, Chance ML, Kemp SJ, Watts PC, Noyes HA (2004) *Leishmania donovani* is the only cause of visceral leishmaniasis in East Africa; previous descriptions of *L. infantum* and "*L. archibaldi*" from this region are a consequence of convergent evolution in the isoenzyme data. Parasitology 129: 399–409.

Jiménez M, González E, Iriso A, Marco E, Alegret A, Fúster F, Molina R (2013) Detection of *Leishmania infantum* and identification of blood meals in *Phlebotomus perniciosus* from a focus of human leishmaniasis in Madrid, Spain. Parasitol Res 112: 2453–2459.

Jiménez M, González E, Martín-Martín I, Hernández S, Molina R (2014) Could wild rabbits (*Oryctolagus cuniculus*) be reservoirs for *Leishmania infantum* in the focus of Madrid, Spain?. Vet Parasitol 202: 296-300.

Kouam MK, Diakou A, Kanzoura V, Papadopoulos E, Gajadhar AA, Theodoropoulos G (2010) A seroepidemiological study of exposure to *Toxoplasma*, *Leishmania*, *Echinococcus* and *Trichinella* in equids in Greece and analysis of risk factors. Vet Parasitol 170: 170–175.

Kuhls K, Mauricio I, Pratlong F, Presber W, Schonian G (2005) Analysis of ribosomal DNA internal transcribed spacer sequences of the *Leishmania donovani* complex. Microbes Infect 7: 1224–1234.

Leite RS, Ferreira S de A, Ituassu LT, de Melo MN, de Andrade ASR (2010) PCR diagnosis of visceral leishmaniasis in asymptomatic dogs using conjunctival swab samples. Vet Parasitol 170: 201–206.

Millán J, Ferroglio E, Solano-Gallego L (2014) Role of wildlife in the epidemiology of *Leishmania infantum* infection in Europe. Parasitol Res 113: 2005–2014.

Molina R, Jiménez MI, Cruz I, Iriso A, Martín-Martín I, Sevillano O, Melero S, Bernal J (2012) The hare (*Lepus granatensis*) as potential sylvatic reservoir of *Leishmania infantum* in Spain. Vet Parasitol 190: 268–271.

Moreno I, Álvarez J, García N, de la Fuente S, Martínez I, Marino E, Toraño A, Goyache J, Vilas F, Domínguez L, Domínguez M (2014) Detection of anti-*Leishmania infantum* antibodies in sylvatic lagomorphs from an epidemic area of Madrid using the indirect immunofluorescence antibody test. Vet Parasitol 199: 264–267.

Ntais P, Sifaki-Pistola D, Christodoulou V, Messaritakis I, Pratlong F, Poupalos G, Antoniou M (2013) Leishmaniases in Greece. Am J Trop Med Hyg 89: 906–915

Parvizi P, Moradi G, Akbari G, Farahmand M, Ready PD, Piazak N, Assmar M, Amirkhani A (2008) PCR detection and sequencing of parasite ITS-rDNA gene from reservoirs host of zoonotic cutaneous leishmaniasis in central Iran. Parasitol Res 103: 1273–1278.

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. Bioinforma Oxf Engl 14: 817–818.

Ruiz-Fons F, Ferroglio E, Gortázar C (2013) *Leishmania infantum* in free-ranging hares, Spain, 2004-2010. Euro Surveill 18(30):pii=20541.

Schönian G, Akuffo H, Lewin S, Maasho K, Nylén S, Pratlong F, Eisenberger CL, Schnur LF, Presber W (2000) Genetic variability within the species *Leishmania aethiopica* does not correlate with clinical variations of cutaneous leishmaniasis. Mol Biochem Parasitol 106: 239–248.

Sobrino R, Ferroglio E, Oleaga A, Romano A, Millán J, Revilla M, Arnal MC, Trisciuoglio A, Gortázar C (2008) Characterization of widespread canine leishmaniasis among wild carnivores from Spain. Vet. Parasitol 155: 198-203.

Souza TD, Turchetti AP, Fujiwara RT, Paixão TA, Santos RL (2014) Visceral leishmaniasis in zoo and wildlife. Vet Parasitol 200: 233–241

Tabachnick BG, Fidell LS (2007) Using multivariate statistics. Allyn & Bacon/Pearson Education, Boston.

Wang J-Y, Gao C-H, Yang Y-T, Chen H-T, Zhu X-H, Lv S, Chen S-B, Tong S-X, Steinmann P, Ziegelbauer K, Zhou X-N (2010) An outbreak of the desert sub-type of zoonotic

visceral leishmaniasis in Jiashi, Xinjiang Uygur Autonomous Region, People's Republic of China. Parasitol Int 59: 331–337.

Yang B-B, Guo X-G, Hu X-S, Zhang J-G, Liao L, Chen D-L, Chen J-P (2010) Species discrimination and phylogenetic inference of 17 Chinese *Leishmania* isolates based on internal transcribed spacer 1 (ITS1) sequences. Parasitol Res 107: 1049–1065.

Zemanová E, Jirků M, Mauricio IL, Miles MA, Lukes J (2004) Genetic polymorphism within the *Leishmania donovani* complex: correlation with geographic origin. Am J Trop Med Hyg 70: 613–617.

# CHAPTER III

## SEROLOGICAL AND MOLECULAR INVESTIGATION OF SELECTED PARASITIC PATHOGENS IN EUROPEAN BROWN HARE (*LEPUS EUROPAEUS*) IN GREECE; INFERRING THE ECOLOGICAL NICHE OF *TOXOPLASMA GONDII* AND *LEISHMANIA INFANTUM* IN HARES

## Abstract

The occurrence of infection and/or exposure to *Toxoplasma gondii, Neospora caninum* and *Leishmania infantum* was investigated in European brown hares (*Lepus europaeus*) hunter harvested during two-year hunting periods in northern and central Greece. Geographical information system (GIS) together with the Ecological Niche Model (ENM) were used to define the geographical distribution of seropositive hares in relation to environmental parameters and to identify high-risk areas for hare exposure. Molecular analysis showed that 3.8% and 9.6% of the examined hares were *N.caninum* and *L.infantum* infected respectively, while, 5.7%, 0.95% and 12.4% of the hares reacted positively for the presence of antibodies against *T.gondii, N.caninum* and *L.infantum* respectively. None of the examined hares was PCR positive for *T.gondii*. Mixed exposure against both *T. gondii* and *L. infantum* was found in 2.9% of the hares examined. Rainfall indices and land uses were significantly influential for hare exposure to *T.gondii* and *L.infantum*. This is the first molecular and serological survey of protozoan pathogens in European brown hare in Greece. Furthermore, we report the environmental parameters related to hare seropositivity and we present a risk map for hare exposure to *T.gondii* and *L.infantum* in northern and central Greece. The ecological niche of *T. gondii* and *L. infantum* in hares herein presented could be applied in other regions with similar environmental and climatic conditions.

## Introduction

The European brown hare (*Lepus europaeus*) presents a remarkable ecological plasticity and it is thus encountered in very different kinds of habitat (Alves et al., 2008). The epizootiological importance of this species stems for the fact that it maintains the so-called home range (Kunst et al., 2001), being thus prone to many infectious diseases of natural nidality while also being a susceptible bio-indicator of environmental changes (Treml et al., 2007). Besides, hare share the same living space with other wild and free-ranging animals including canids, felids and large birds of prey, which comprise its natural predators (Alves et al., 2008) and hunting grounds with domestic dogs.

Domestic and free-ranging felids are the only definitive known hosts for *T. gondii*, able to produce and shed the environmentally-resistant stage, oocysts, in their feces. Hares are regarded as exceptionally susceptible to primary infection (Gustafsson et al., 1997; Jokelainen et al., 2011). Infected hares can represent a potential source of *T. gondii* for other animals, especially for carnivores but also for humans (Bártová et al., 2010).

Canids including dog, coyotes (*Canis latrans*) (Gondim et al., 2004), Australian dingoes (*Canis lupus dingo*) (King et al., 2010) and gray wolves (*Canis lupus*) (Dubey and Schares, 2011) are natural definitive hosts for *N. caninum*, shedding oocysts in their faeces while wild herbivores are intermediate hosts in the sylvatic cycle of the parasite (Gondim, 2006). Regarding hares, they are considered a potential source of *N. caninum* infection especially for carnivores (Bártová et al., 2010).

The domestic dog is the most important vertebrate host and the main reservoir for *L. infantum.* The role of the cat has yet to be defined (Giannakopoulos et al., 2017) while several wild mammal species are susceptible to *L. infantum* infection (Souza et al., 2014). Recently, the unexpected role of hares as a sylvatic reservoir of *L. infantum* was revealed through the investigation of the outbreak of human leishmaniasis cases in the south-western Madrid region (Molina et al., 2012) while *Leishmania* infection in hares was also reported in Northern Greece (Tsokana et al., 2015).

Geographic Information Systems (GIS) have been used in several studies for surveillance and monitoring of different pathogens (Jores et al., 2008; Valiakos et al., 2014), identification of environmental parameters related to occurrence of pathogens in domestic and wild animals (Giannakopoulos et al., 2016; Miller et al., 2002), risk assessment (Valle and Tucker Lima, 2014) and creation of risk maps for the presence

of infected individuals (Giannakopoulos et al., 2016). MaxEnt a method that uses presence-only data (Phillips et al., 2006) has been applied successfully to predict the spread of important infectious diseases such as Leishmaniasis, dengue fever and monkeypox (Arboleda et al., 2009; Giannakopoulos et al., 2016; Nakazawa et al., 2013). The epidemiological studies on wildlife are scanty in Greece while to our knowledge, there are limited data available on parasitic pathogens in the hare population. The aims of this study were a) to define the infection and exposure status of European Brown hares from northern and central Greece against *T.gondii, N.caninum* and *L.infantum*, b) to identify the environmental parameters related with hare exposure to *T.gondii* and *L.infantum* using GIS and ENM analysis c) to create risk maps and to define the high-risk areas for hare exposure to these pathogens.

## Materials and Methods

### Hare samples

From a total of 105 free-ranged hares, 105 serum samples and 52 liver samples were collected by hunters and foresters during two-year hunting seasons in northern and central Greece. Blood samples were collected from the heart or the thoracic cavity of the shot hares, they were centrifuged and sera was stored at -20°C pending examination for antibodies against *T.gondii, N.caninum* and *L.infantum*. The hare liver samples were stored at -20˚C and they were submitted still frozen to the Laboratory of Microbiology and Parasitology, University of Thessaly, Greece. Data on gender were recorded, when possible, for the subjects included in the study. The location data of hares were marked with handheld Global Positioning System (GPS) Garmin units in the field.

### Serological investigation

In total, 105 serum samples were tested for antibodies by indirect fluorescence antibody test kits (IFAT) using different commercial agent specific slides to detect antibodies against *T.gondii, N.caninum*, (Agrolabo, Scarmagno, Italy) and *L.infantum* (Fuller Laboratories Fullerton, California, USA) following the manufacturer's instructions. For all IFATs, a fluorescein isothiocyanate conjugated sheep anti rabbit IgG (BioFX Laboratories, Owings Mills, USA) was used. Two-fold dilutions of the samples

were prepared in phosphate buffered saline (PBS), starting from 1:20 as a basic dilution to detect antibodies against *T.gondii* and *N.caninum* and 1:25 to detect antibodies against *L.infantum* until the end-point titer; titers ≥1:40 for *T.gondii* and *N.caninum* and ≥ 1:50 for *L.infantum* were considered positive. Positive and negative controls provided by the manufacturers were included in all the tests.

## Molecular investigation

Total genomic DNA extraction was performed using a commercially available DNA extraction kit (Thermo Scientific GeneJET Genomic DNA Purification Kit, Thermo Fisher Scientific, USA) according to the manufacturer's protocol in 52 liver hare samples. The purified DNA was stored at -20˚C. Previously described conventional PCRs were carried out for detecting DNA of *T.gondii* (Homan et al., 2000), *N.caninum* (Müller et al., 1996) and *L.infantum* (El Tai et al., 2001; Leite et al., 2010). Positive and negative controls were included in each PCR run. The PCR products were visualized under UV-light, after 2% agarose gel electrophoresis and staining with ethidium bromide (0.5 μg/ml).

Amplicons from PCR positive hares were sequenced. The positive PCR products were purified using the PureLink PCR purification kit (Invitrogen, Carlsbad, CA, USA) and were bidirectionally sequenced using the fluorescent BigDye Terminator Cycle sequencing kit v3.1 (Applied Biosystems, Foster City, USA), followed by fragment separation with a 3730xl DNA analyzer (Applied Biosystems, Foster City, USA).

## Geographical Information System (GIS)

Altitude was extracted from a digital elevation model (DEM) with a spatial resolution of 1 square kilometer. Land uses were determined from the Corine Land Cover 2000 database (European Environment Agency-EEA) and the ArcGIS online application. All data layers were converted to a common projection, map extent and resolution. ArcGIS 10.1 GIS software (ESRI, Redlands, CA, USA) was used for data analysis.

European brown hare (*Lepus europaeus*) population distribution was obtained from a GIS data base from the GIS research unit of the Department of Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Thessaly, Karditsa, Greece.

## Environmental variables

Climatic variables were derived from the WorldClim website version 1.4. (Hijmans et al., 2005). Wind speed was retrieved from the GeoPortal of the Hellenic Regulatory Authority for Energy. Digital elevation model (altitude) was extracted from CGIAR-CSI GeoPortal. Hydrological data were extracted from HydroSHEDS. Land uses (habitat types) derived from the Corine Land Cover 2000 database (European Environment Agency, Copenhagen, Denmark. The normalized difference vegetation index (NDVI), an indicator of the greenness of the biomes per month, was extracted from the Copernicus European earth monitoring program (FDC, Vincennes, France).

## Environmental Niche Model (ENM)

ENM or species distribution modelling programs, e.g. MaxEnt (Phillips et al., 2006), enable the description of the probability of a species distribution range. An algorithm for maximum entropy is used by MaxEnt to calculate the realized niche of the species and the occurrence probability. Sites with similar environments to those where a species has already been observed are identified by MaxEnt. The identification of these potential distribution areas is made using species presence points (e.g. *L.infantum* seropositive hares in our study), as well as the environmental variables of the study area.

First, a niche was defined based on the environmental values that correspond to 'presence' data (in this case, *T.gondii* and *L.infantum* seropositive hares). Then, similarities between environmental values at a specific cell and those of the niche of *T.gondii* and *L.infantum* seropositive hares were calculated for each raster cell in the study area. This information was used for the calculation of the probability of *T.gondii* and *L.infantum* seropositive hare occurrence in each raster cell.

In the MaxEnt modelling, the pixels of the study area define the area where the distribution of the MaxEnt probability is defined. Pixels with occurrence records constitute the sample points and the features are environmental parameters (climatic, vegetation, topographic etc.). MaxEnt method requires presence-only data, utilizes both continuous and categorical data and includes efficient deterministic algorithms and mathematical definitions (Phillips et al., 2006). *T.gondii* and *L.infantum* seropositive hares were used as occurrence points for the ENM procedure. Maximum entropy

modelling (MaxEnt software version 3.3·3) was used to predict the appropriate ecological niches for hares (Phillips et al., 2006). The goodness of fit of the model predictions was evaluated by the mean area under the curve (AUC) of the receiver operating characteristic curve. We used the Jackknife procedure to reduce the number of environmental variables to only those that showed a substantial influence on the model. At first, all the available environmental parameters were used for the analysis. Two sets of parameters were created with a very large difference in their percentage of participation in the prediction process. Parameters with a participation rate lower than 2% were omitted from the final model based on which, the potential areas for the spatial distribution of *T.gondii* and *L.infantum* seropositive hares were determined. According to Ceccarelli et al., 2015 we repeated the test with the Jackknife test until all the remaining variables have a positive effect on the total gain. High-risk areas were considered those with >63% probability of seropositivity for *T.gondii* and *L.infantum*.

### Statistical analysis

The exact binomial test established confidence intervals (CI) with a 95% confidence level. The chi-square and Fisher's exact tests were used to compare percentages of seropositivity for *T.gondii* as well as *L.infantum* among the different study areas (Thessaly, central Macedonia and eastern Macedonia and Thrace). A *p value* <0.05 was considered as statistically significant. Analyses were performed with IBM SPSS V.22.0.

### Ethics

The hare serum and liver samples included in the study, represent material collected opportunistically (no active capture, killing and sampling of wild animals specifically for this study was performed) from animals hunter-harvested by members of the Greek Hunting Federation of Macedonia and Thrace and the Greek Hunting Federation of Thessaly and Sporades, during the hunting seasons, according to the prerequisites of the Greek Legislation and submitted to our laboratory for Passive Wildlife Disease surveillance. Thus, special approval was not necessary and steps to ameliorate suffering were not applicable in this study. Research on animals as defined in the EU Ethics for Researchers document (European Commission, 2007, Ethics for Researchers -

Facilitating Research Excellence in FP7, Luxembourg: Office for Official Publications of the European Communities, ISBN 978-92-79-05474-7) is not a part of the study.

## Results

### Serological investigation

In total, antibodies against *T. gondii* were detected in six (5.7%, 95% CI: 2.1-12) hare serum samples; three (9.6%, 95% CI: 2-25.7) in Thessaly, one (2.6%, 95% CI: 0.07-13.8) in central Macedonia and two (5.5%, 95% CI: 0.6-18.6) in eastern Macedonia and Thrace. Antibodies against *N. caninum* were detected in only one (0.95%, 95% CI: 0-5.2) hare serum sample from central Macedonia. Antibodies against *L. infantum* were detected in 13 (12.4%, 95% CI: 6.8-20.2) hare serum samples; three (9.7%, 95% CI: 2-25.8) in Thessaly, five (13.2%, 95% CI: 4.4-28.1) in central Macedonia and five (13.9%, 95% CI: 4.7-29.5) in eastern Macedonia and Thrace (Table 1). Antibodies against at least one of the pathogens examined were detected in 17 hares (16.2%, 95% CI: 9.7-24.7). Mixed exposure (concurrent presence of antibodies against both *T. gondii* and *L. infantum*) was found in three hares (2.9%, 95% CI: 0.6-8.1); one hare in each one of the study areas (Thessaly, central Macedonia, eastern Macedonia and Thrace) (Table 1).

Table 1. The prevalence of antibodies against *T.gondii, L.infantum* and *N.caninum* in European brown hares from central and northern Greece

| Region | Hare serum samples | *T.gondii* | | | *L.infantum* | | | Mixed exposure to *T. gondii* and *L.infantum* | *N. caninum* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Positive | Titre | *P value* | Positive | Titre | *P value* | | Positive | Titre |
| Thessaly | 31 | 3 (9.6%) | 1:40-1:160 | | 3 (9.7%) | 1:50-1:200 | | 1 (3.2%) | 0 | - |
| Central Macedonia, northern Greece | 38 | 1 (2.6%) | 1:40 | | 5 (13.2%) | 1:50-1:100 | | 1 (2.6%) | 1 (2.6%) | 1:320 |
| Eastern Macedonia and Thrace, northern Greece | 36 | 2 (5.5%) | 1:40-1:160 | 0.45 | 5 (13.9%) | 1:50-1:200 | 0.85 | 1 (2.8%) | 0 | - |
| Total | 105 | 6 (5.7%) | 1:40-1:160 | | 13 (12.4%) | 1:50-1:200 | | 3 (2.9%) | 1 (0.95%) | |

Titers of antibodies against *T. gondii* ranged from 1:20 to 1:160; serum titers of 1:20 in four hares, 1:40 in three hares and 1:160 in three hares. Titer of antibodies against *N. caninum* in the one seropositive hare found in this study was 1:320. Titers of antibodies against *L.infantum* ranged from 1:25 to 1:200; serum titers of 1:25 in two hares, 1:50 in five hares, 1:100 in one hare and 1:200 in seven hares.

In overall, records on the gender were available for 67 out of the 105 subjects (36 males and 31 females). Out of the six *T. gondii* seropositive hares, four were males (66%). All the *L. infantum* seropositive hares with gender records (n=9/13) were males.

## Molecular investigation

*N.caninum* DNA was detected in two hare liver samples (one male and one female hare) from Thessaly, central Greece (3.8%, 95% CI: 1-12.9). *Leishmania* DNA was detected in five hare liver samples (9.6%, 95% CI: 4.1-20.6); two from central Greece (7.4%, 95% CI: 2-23.3) and three from central Macedonia (20%, 95% CI: 7 -45.1). All the hare liver samples (n=10) from eastern Macedonia and Thrace were PCR negative. Among the *Leishmania* PCR positive hares, gender records were available only for one hare which was a female individual. None of the examined hare liver samples was found positive for *T.gondii* (Table 2). The five *T.gondii* and the seven *L.infantum* seropositive hares for which liver samples were available, were PCR negative. As for the only *N.caninum* seropositive hare, no liver sample was available for molecular examination.

One *N.caninum* and two *Leishmania* amplicons were sequenced and the sequences have been deposited in GenBank under accession numbers MH568687 and KY379079-KY379080 respectively. Sequence analysis revealed 96-98% homology with other *N.caninum* sequences and 99% homology with *L. infantum*.

Table 2. Molecular investigation for *T.gondii*, *L.infantum* and *N.caninum* DNA in liver samples from European brown hares from central and Northern Greece.

| Region | Hare liver samples | *T.gondii* Positive | *L.infantum* Positive | *P value* | *N.caninum* Positive |
|---|---|---|---|---|---|
| Thessaly, central Greece | 27 | 0 | 2 (7.4%) | | 2 (7.4%) |
| Central Macedonia, northern Greece | 15 | 0 | 3 (20%) | 0.31 | 0 |
| East Macedonia and Thrace, northern Greece | 10 | 0 | 0 | | 0 |
| Total | 52 | 0 | 5 (9.6%) | | 2 (3.8%) |

## Geographical Information System (GIS)

The spatial distribution of *T. gondii* and *L.infantum* seropositive hares is shown in Fig. 1 along with the geographical distribution of European brown hare population in the study area.

The *T. gondii* seropositive hares were found in agroforestry formations (50%), in shrubland with pastures (40%) and in conifer forests (10%) with a mean altitude of 250.33m above sea level (range 55–584±222.83 SD), while the mean distance from villages and towns was 2.764 m (range 1931–4368±860.80 SD). The mean distance from livestock farms was 1164.13 m (range 223.60–2959.85±898.96 SD). The mean cattle, goat and sheep density in the areas where *T. gondii* seropositive hares were found was 8.43 animals/km$^2$ (range 0.10–13.89±4.33 SD), 37.29 animals/km$^2$ (range 18.45–65.29±15.69 SD) and 151.76 animals/km$^2$ (range 1.02–329.20±109.76 SD) respectively.

The *T. gondii* seronegative hares were found in agroforestry formations (35%), in shrubland with pastures (45%) and in broadleaved forest (20%) with a mean altitude of 441.06 m above sea level (range 56–1652 ± 363.23 SD), while the mean distance from villages and towns was 3.391 m (range 670–11634±2195.39 SD). The mean distance

from livestock farms was 1291.57 m (range 100.80–4440.72±886.46 SD). The mean cattle, goat and sheep density in the areas where *Toxoplasma* seronegative hares were found was 7.40 animals/km$^2$ (range 0.03–19.86±5.38 SD), 32.33 animals/km$^2$ (range 9.76–81.93±16.64 SD) and 67.14 animals/km$^2$ (range 0,40–346.90±81.63 SD) respectively.

The *L.infantum* seropositive hares were found in shrubland with pastures (70%) and in broadleaved forests in combination with cultivations (30%) with a mean altitude of 236.07m above sea level (range 59–518±141.63 SD), while the mean distance from villages and towns was 2.824 m (range 1140–6200±1522.14 SD).

The *L.infantum* seronegative hares were found in shrubland with pastures (60%), in broadleaved forests in combination with meadows (25%) and in agroforestry formations (15%) with a mean altitude of 454.48m above sea level (range 55–1652±370.58 SD), while the mean distance from villages and towns was 3.381 m (range 670–11634±2152.39 SD).

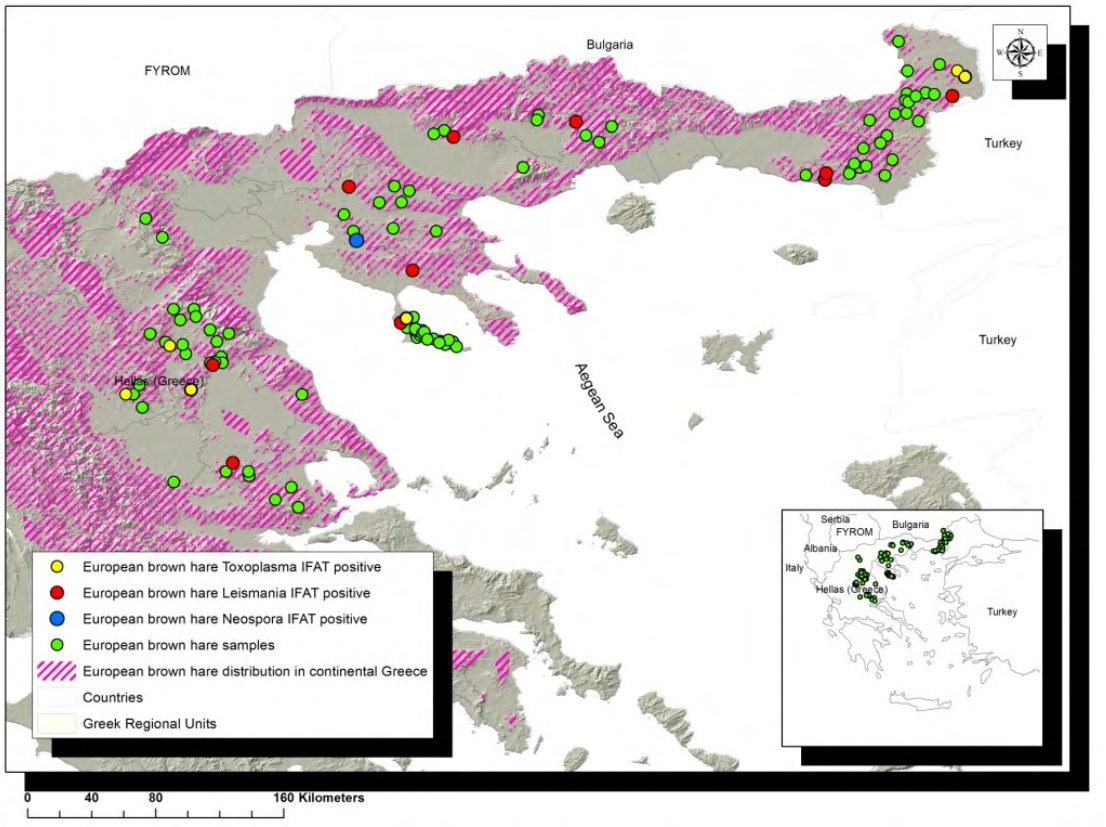


**Figure 1. Map of Greece showing the geographical distribution of the *T.gondii*, *N.caninum* and *L.infantum* seropositive hares.** Map of Greece showing the geographical distribution of the European Brown hare in continental Greece and the *T.gondii*,

*N.caninum* and *L.infantum* seropositive hares found in central and northern Greece. Green dots indicate the hare samples collected in the study area. Yellow, blue and red dots indicate the *T.gondii*, *N.caninum* and *L.infantum* seropositive hares respectively.

### Predictive ENM for *T. gondii* seropositive hares

The contribution of the environmental variables to the MaxEnt model analysed in this study for *T. gondii* seropositive hares are shown in Table 3. Jackknife of regularized training gain test for *T. gondii* seropositive hares in the study area is shown in Fig. 2. The environmental variable with the highest gain when used in isolation was Precipitation of Wettest Month. The variable that decreased gain the most when omitted, was Corine Land Cover-habitat types. In particular, the presence of *T.gondii* seropositive hares was associated with the following categories; coniferous forests, mixed forest, non-irrigated arable land and natural grasslands. Regularized training gain (sum of the likelihood of the data plus a penalty function) was 1.367, training AUC was 0.959 and unregularized training gain was 2.745. Other significant variables according to the MaxEnt model were altitude, the Normalized difference vegetation index in April, livestock density and the Mean Temperature of Wettest Quarter. The potential distribution of the *T.gondii* exposed hares in the study area together with the recognized high risk areas for hare exposure to *T.gondii* are presented in Fig.3.

Table 3. The relative contributions of the environmental variables to the Maxent model for the exposure of European brown hare to *T.gondii*.

| Variable | Percent contribution | Permutation importance |
|---|---|---|
| Precipitation of Wettest Month | 27 | 6.2 |
| Corine Land Cover-habitat types | 24 | 22.2 |
| Altitude | 18.9 | 16.4 |
| Normalized difference vegetation index-April month | 14 | 15.2 |
| Livestock density | 12.1 | 14.5 |
| Mean Temperature of Wettest Quarter | 4.1 | 25.4 |

| | | |
|---|---|---|
| Farms distance | 0 | 0 |
| Population density | 0 | 0 |
| Normalized difference vegetation index-September month | 0 | 0 |
| Wind speed | 0 | 0 |

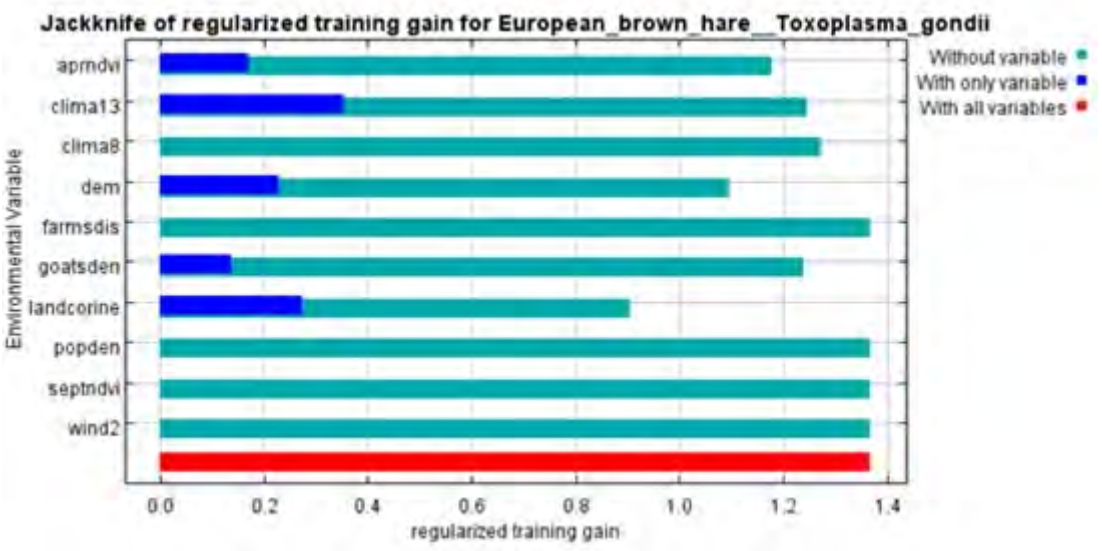


Figure 2. Jackknife of regularized training gain test for *T. gondii* seropositive hares in the study area. Aprndvi: NDVI in April, clima 13: Total precipitation of wettest month, clima 8: Mean temperature of wettest quarter, dem: altitude, farmsdis: distance from farms, goatsden: livestock density, lancorine: Corine land cover-habitat types, popden: population density, septndvi: NDVI in September, wind2: wind speed

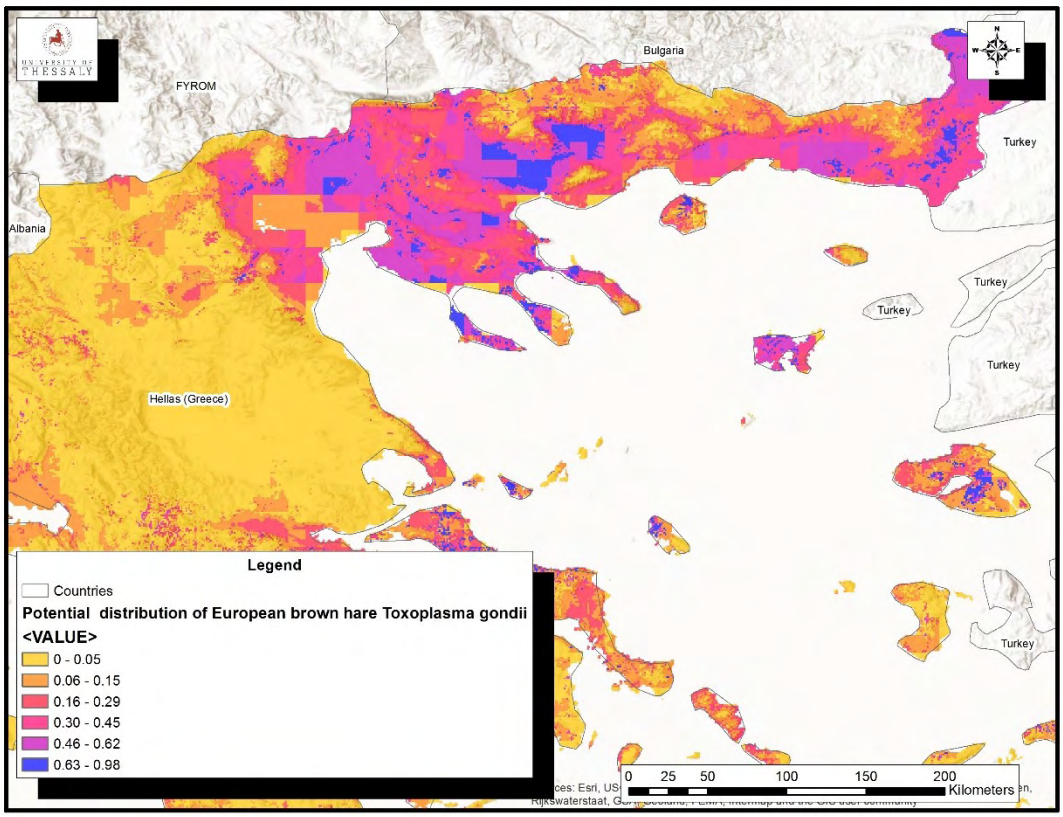


Figure 3. Map of the study area showing the probability for the presence of *T.gondii* exposed hares together with the recognized high risk areas for hare exposure to *T.gondii*

## Predictive ENM for *L.infantum* seropositive hares

The contribution of the environmental variables to the MaxEnt model analysed in this study for *L.infantum* seropositive hares are shown in Table 4. Jackknife of regularized training gain test for *L.infantum* seropositive hares in the study area is shown in Fig. 4. The environmental variable with the highest gain when used in isolation was Precipitation of the Wettest Month. The variable that decreased gain the most when omitted, was Corine Land Cover-habitat types. In particular, the presence of *L.infantum* seropositive hares was associated with the following categories; natural grasslands, coniferous forests, land principally occupied by agriculture, with significant areas of natural vegetation and non-irrigated arable land. Regularized training gain (sum of the likelihood of the data plus a penalty function) was 1.774, training AUC was 0.976 and unregularized training gain was 2.648. Other significant variables according to the

MaxEnt model are Minimum Temperature of Coldest Month, altitude, livestock density, Mean Temperature of Wettest Quarter and wind speed. The potential distribution of the *L.infantum* exposed hares in the study area together with the recognized high risk areas for hare exposure to *L.infantum* are presented in Fig.5

Table 4. The relative contributions of the environmental variables to the Maxent model for the exposure of European brown hare to *L.infantum*.

| Variable | Percent contribution | Permutation importance |
|---|---|---|
| Precipitation of Wettest Month | 29.6 | 14.4 |
| Corine Land Cover-habitat types | 21.3 | 12.7 |
| Min Temperature of Coldest Month | 20.3 | 3.6 |
| Altitude | 13 | 51.3 |
| Livestock density | 9.2 | 0 |
| Mean Temperature of Wettest Quarter | 4.9 | 15.6 |
| Wind speed | 1.6 | 1.7 |
| Normalized difference vegetation index-November month | 0 | 0 |
| Normalized difference vegetation index-August month | 0 | 0.6 |
| Precipitation of Wettest Quarter | 0 | 0 |
| Normalized difference vegetation index-April month | 0 | 0 |
| Farms distance | 0 | 0 |
| Population density | 0 | 0 |

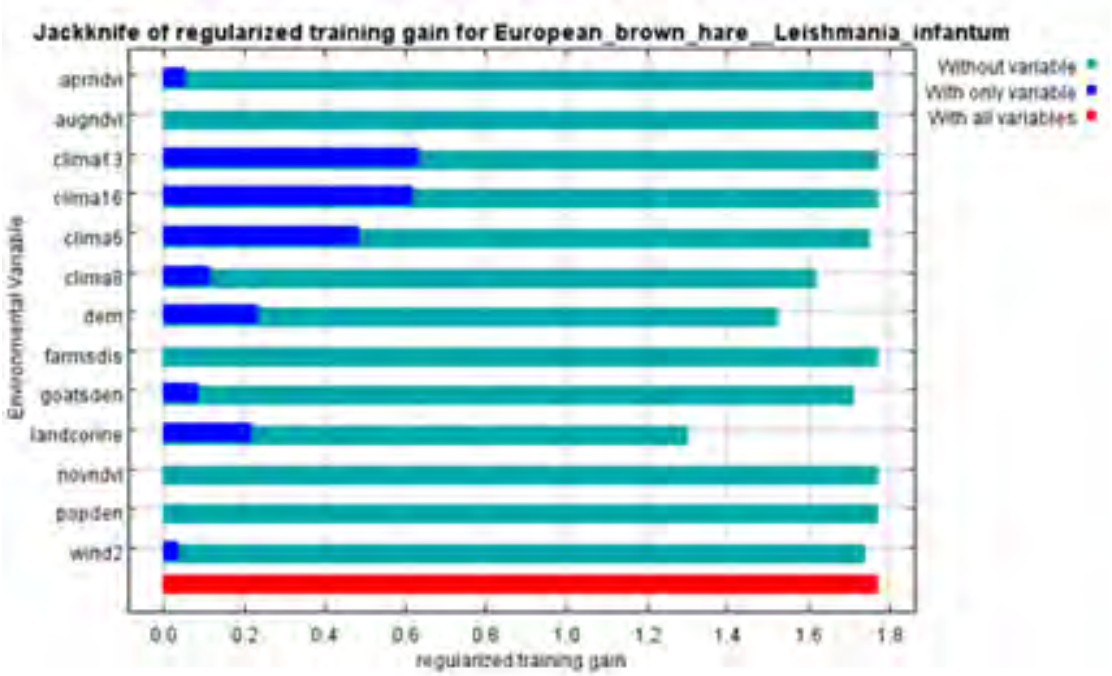


Figure 4. Jackknife of regularized training gain test for *L.infantum* seropositive hares in the study area. Aprndvi: NDVI in April, augndvi: NDVI in August, clima 13: Total precipitation of wettest month, clima 16: Total precipitation of wettest quarter, clima 6: Minimum temperature of coldest month, clima 8: Mean temperature of wettest quarter, dem: altitude, farmsdis: distance from farms, goatsden: livestock density, lancorine: Corine land cover-habitat types, novndvi: NDVI in November, popden: population density, wind2: wind speed

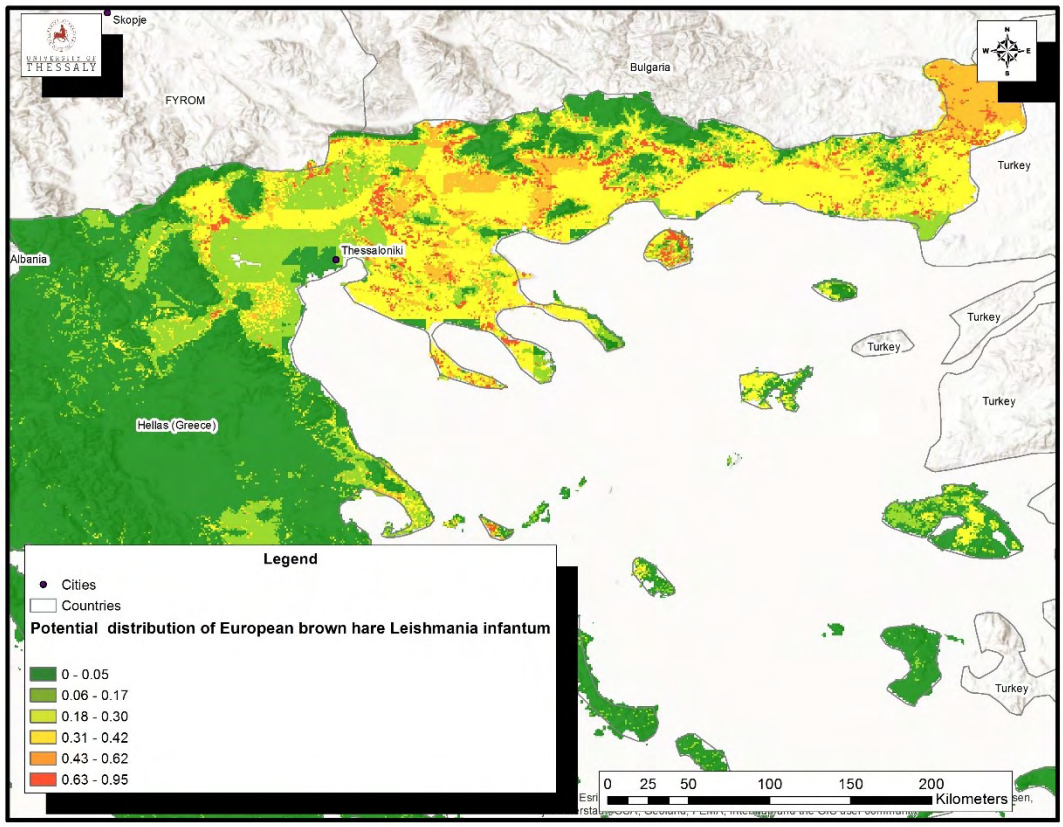


Figure 5. Map of the study area showing the probability for the presence of *L.infantum* exposed hares in the study area together with the recognized high risk areas for hare exposure to *L.infantum*

## Statistical analysis

No statistically significant difference was found among the seroprevalence for *T.gondii* ($p$=0.45) as well as *L.infantum* ($p$=0.85) in Thessaly, central Macedonia and eastern Macedonia and Thrace (Table 1). Similarly, no statistically significant difference was found among the *L.infantum* DNA prevalence in the three different regions of Greece ($p$=0.31) (Table 2).

## Discussion

This study provides evidence of *T.gondii* (5.7%), *N.caninum* (0.95%) and *L.infantum* (12.4%) exposure in hares from central and northern Greece. Antibodies against at least one of the pathogens examined were detected in 16.2% while concurrent presence of antibodies against both *T.gondii* and *L.infantum* was found in 2.9% of the hares included in the study. The occurrence of *N.caninum* (3.8%) and *L.infantum* (9.6%)

infection and the lack of evidence for *T.gondii* infection in the hare population examined are justified through molecular examination. Interestingly, none of the *T.gondii* and *L.infantum* seropositive hares was found PCR positive while the infection status of the only *N.caninum* seropositive hare is unknown due to the lack of liver sample from this individual. Moreover, ENM from MaxEnt (Phillips et al., 2006) was used to identify the environmental variables associated with hare exposure to *T.gondii* and *L.infantum* and to recognize high risk areas for hare exposure through the creation of a risk map for the regions of central and northern Greece. The analysis revealed that the precipitation of the wettest month contributes to the highest per cent to define environmental niche profiles for hare exposure to both pathogens in the study area. Another important environmental parameter contributing to both prediction models was land cover (habitat types).

The exceptional susceptibility of hares to *T.gondii* primary infection (Gustafsson et al., 1997; Lindsay and Dubey, 2014) has been supported by the low prevalence of latent chronic or subclinical infections in hares (Gustafsson and Uggla, 1994). However, high seroprevalence in hares (Frölich et al., 2003) has been also reported suggesting that they can survive *T. gondii* infection in the wild. Hares get infected through consumption of food or water contaminated with oocysts shed by domestic cats or free-ranging felids with which they share the same habitat. Seroprevalence of *T. gondii* in hares varies among European countries ranging from 0-46% (Aubert et al., 2010; Bartova and Sedlak, 2012; Catar, 1972; Edelhofer et al., 1989; Frölich et al., 2003; Gustafsson and Uggla, 1994; Poli et al., 1987). Although different serological techniques were used in the above-mentioned studies and thus a direct comparison of prevalences is not possible, the differences in the serological status of hare population against *T. gondii* across European countries are indeed highlighted. In Greece, the seroprevalence of *T. gondii* in sheep and goats ranged from 25% in central Greece (Kantzoura et al., 2013) to 49% and 31% respectively (Tzanidakis et al., 2012) in Northern Greece and 54% and 61% in various regions of Greece (Diakou et al., 2013). An average seroprevalence of 27% was reported in swine herds in the mainland Greece (Papatsiros et al., 2016) and 1.8% in equids in four regions in Greece (Kouam et al., 2010). As far as it concerns the wildlife, (Touloudi et al., 2015) reported a seroprevalence of 5.2% in wild boars. Although a direct comparison can be made only with the studies that used IFAT for the

detection of antibodies against *T.gondii,* in overall, the results of our study (5.7%) are in agreement with previous studies conducted in Austria (2% by IFAT) (Edelhofer et al., 1989), Italy (6.4% by indirect agglutination test) (Poli et al., 1987) and France (9% by MAT) (Aubert et al., 2010). The seroprevalence herein reported in hares is in great agreement with the seroprevalence reported previously in Greek wild boars (5.2%) (Touloudi et al., 2015). In our study, *T.gondii* DNA was not detected in any of the hare liver samples examined which is in agreement with a previous study on the Iberian hare (Fernández-Aguilar et al., 2013). Although liver has been previously proposed as a tissue of choice, *T.gondii* DNA could be present in other tissues such as brain or heart as it has been observed in subclinical infections in other animal species (Dubey, 2010; Fernández-Aguilar et al., 2013; Jokelainen, 2012).

Precipitation of the wettest month was the environmental parameter contributing to the highest per cent to define environmental niche profiles for hare exposure to *T.gondii* in the study area. This finding is concordant with previous observations that increased prevalence in cats (Afonso et al., 2010), rabbits (*Oryctolagus cunniculus*) (Almería et al., 2004) and roe deer (*Capreolus capreolus*) (Gamarra et al., 2008) is associated with high levels of rainfall. It has been suggested that precipitation influences the dynamics of oocysts in the environment creating a moist environment that allows for oocysts survival while also it increases the food availability supporting the high densities of hosts (Afonso et al., 2013; Yan et al., 2016), including transport hosts such as arthropods, that require a humid environment, and flies, cockroaches and earthworms which may potentially spread *T. gondii* to other hosts through ingestion of oocyst (Bettiol et al., 2000; Frenkel et al., 1995; Yan et al., 2016).

The presence of *T.gondii* seropositive hares was associated with coniferous and mixed forests, non-irrigated arable land and natural grasslands. Forests may be linked with the presence of significant wildcat and wildcat-domestic hybrid populations (Germain et al., 2008) resulting in increased soil contamination from their feaces and higher probability for hare exposure to *T.gondii*. Additionally, such land types are characterized by moderate to high annual precipitation thus influencing the dynamics of oocysts survival and dispersal as mentioned above. In areas with arable lands, irrigation of soil and soil disturbance during agricultural practices increases oocyst survival and distribution while food availability affects the population density of

important hosts such as rodents (Gotteland et al., 2014; Smallbone et al., 2017; Yan et al., 2016). Natural grasslands provide moist and shaded microenvironments which facilitate survival of oocysts (Du et al., 2012) and the soil presents high permeability and it is rich in organic matter, both characteristics being beneficial for the existence of transport hosts (Bettiol et al., 2000; Frenkel et al., 1995; Gao et al., 2016; Yan et al., 2016).

Although not recognized as leading factors, altitude, normalized difference vegetation index in April, livestock density and temperature were important for model development. Altitude is well related with rainfall with greater rainfall at higher latitudes and decreased precipitation at lower latitudes (Yan et al., 2016). Normalized difference vegetation index in April is an indicator of the greenness of the biomes and possibly affects the food availability for different *T.gondii* hosts (e.g. rodents, migratory birds and arthropods) and consequently their geographical distribution, abundance and migration patterns (Afonso et al., 2013; Ribeiro et al., 2015; Yan et al., 2016). The livestock density, is directly linked to livestock farm density; the spatial overlap between definitive and intermediate hosts in a small area, the existence of an open landscape which facilitates contact between domestic and wild animals (Afonso et al., 2013; Simon et al., 2017), the high density of free-ranging cats in farms (Ferreira et al., 2011; Kitts-Morgan et al., 2015; Liberg, 1980) and the soil disturbance by livestock movements (Lehmann et al., 2003) are those farm characteristics that make them important sources of *T. gondii.* The role of farms has been shown for both cats (Afonso et al., 2013) and intermediate hosts (Gotteland et al., 2014; Lehmann et al., 2003; Richomme et al., 2010) as the proportion of infected individuals is related to the density and proximity to farms. Finally, temperature partly determines the oocyst sporulation and affects their survival and infectivity while also it influences the population density of the intermediate and mechanical hosts including rodents which are considered important in *T. gondii* transmission (Afonso et al., 2013; Gotteland et al., 2014; Smallbone et al., 2017; Yan et al., 2016).

Hares are considered an important source of *N. caninum* infection for carnivores (Bártová et al., 2016; Ferroglio and Trisciuoglio, 2003) as they could be continuously infected. This fact is of particular importance for red foxes because hare is a common prey for them; it can make up to 10.7% (Kidawa and Kowalczyk, 2011) to 46% (Ferroglio

and Trisciuoglio, 2003) of their diet component. Although foxes have not been proved as a definitive host (Bartley et al., 2013; Wapenaar et al., 2006), their important role in maintaining *N. caninum* infection in the sylvatic cycle has been suggested due to their dietary habits and their adaptable and opportunistic nature (Bateman and Fleming, 2012) that enables red foxes to be in close contact with domestic dogs and livestock. Moreover, in Greece, when hares are hunted, the carcasses are eviscerated in the field and the offal is given purposely to hunting dogs or left behind, available for consumption by wild animals. Therefore, each hunting season, hunting dogs, wild canids and wild boar have an increased possibility to consume *N. caninum*-infected hare tissues. However, the low seroprevalence (0.95%) and DNA prevalence (3.8%) found in this study indicates a low risk for these animals to be infected through the consumption of infected hares in Greece. As far as it concerns the European brown hare, there are serological data from Czech Republic (39%), Slovakia (4%), Austria (35%) (Bártová et al., 2010) and Italy where hares were imported from Hungary (8.6%) and Slovakia (6.8%) (Ferroglio and Trisciuoglio, 2003). In Greece, seroprevalences of 16.8% and 6.9% in sheep and goats respectively (Diakou et al., 2013) and 15.2% in cattle (Sotiraki et al., 2008) have been reported while the seroprevalence in wild boars was 1.1% (Touloudi et al., 2015). Our results are not in agreement with the studies conducted previously in hares in other European countries. The seroprevalence found in our study is very low and only comparable to the seroprevalence found in wild boars in Greece (Touloudi et al., 2015) indicating the existing although limited exposure of wildlife to *N.caninum* in Greece. To the best of our knowledge there are no data regarding the prevalence of *N. caninum* in domestic dogs and wild canids, including red foxes in Greece. Though it has been suggested that a low prevalence (15.4%) in grey foxes seems to indicate that their prey also have a low prevalence (Lindsay et al., 2001). Since hares, a common prey for red foxes, present such a low *N. caninum* seroprevalence, it could be expected that a low seroprevalence will also be observed in red foxes in Greece. However, this hypothesis remains to be confirmed by further studies.

Data on the role of hare in the epidemiology of *Leishmania* spp come from Spain, Greece and Italy. In Italy the seroprevalence in *Le. europaeus* was 0.9% (Ebani et al., 2016) which is not in agreement with our results (12.4%) (Ebani et al., 2016) albeit a

direct comparison is possible in terms of the serological technique and the cut-off used. Molecular studies conducted previously, revealed 56.3% *Leishmania* infection in *Le. europaeus* in Spain (Ruiz-Fons et al., 2013), 18.52% in Italy (Zanet et al., 2016) and more recently 1.9% in central Italy (Rocchigiani et al., 2018). In northern Greece, the *Leishmania* DNA prevalence reported from 2007-2011 was 23.49% (Tsokana et al., 2015) which is in great agreement with our current findings in the same region (20%). Though, the results of this study should be treated with caution; a small subset of samples was molecularly examined aiming to show the occurrence of infection rather than to determine the DNA prevalence of *L.infantum* infection in this hare population. Studies on *Leishmania* seroprevalence and DNA prevalence have been conducted in Greece in domestic as well as wild animals. Regarding the canine population, the most recent epidemiological studies reported a seropositivity ranging from 20% - 22.09% (Athanasiou et al., 2012; Ntais et al., 2013). In another study, *Leishmania* PCR positive dogs were found in each one of the four prefectures of Thessaly, Central Greece (Giannakopoulos et al., 2016). A seroprevalence of 3.87% was reported in clinically healthy stray cats living in the area of Thessaloniki, Northern Greece (Diakou et al., 2009) while in Central Greece, the most recent study, reported 13.3% *Leishmania* DNA prevalence in stray and owned cats (Giannakopoulos et al., 2017). Regarding other wild animals, specific anti-*Leishmania* IgG antibodies were detected in 54.55% and *Leishmania* DNA was detected in 19.58% of the rodents examined in Northern Greece (Tsakmakidis et al., 2017). Additionally, *Leishmania* DNA prevalence in red foxes examined in the region of Fthiotida which is adjacent to the prefectures of Larissa and Magnesia, central Greece was 59.5% (Karayiannis et al., 2015). Although *Leishmania* infection in hares has been documented previously in Northern Greece (23.49%) (Tsokana et al., 2015), the occurrence of *Leishmania* infection in hares in central Greece and the seroprevalence of *L. infantum* in both regions have not been reported so far. Thessaly, central Greece is a highly endemic region for human and canine leishmaniasis (Giannakopoulos et al., 2016), where the last years an increase of the incidence rate of human leishmaniasis cases was recorded; 82 human Visceral Leishmaniasis cases were officially notified from 2007-2014 with 60 out of the 82 human VL cases being recorded during 2012-2014 (Giannakopoulos et al., 2016). On the other hand, during 2012-2013,

seven human cases were reported in the northern part of our study area (six cases in Central Macedonia and one case in East Macedonia and Thrace (HCDCP).

The occurrence of *L.infantum* infection in hares, cats, dogs, red foxes and humans in central Greece and the adjacent regions, is highly suggestive of an overlapping of sylvatic and domestic transmission cycles in Thessaly, as it has been previously suggested for the region of Thessaloniki, northern Greece (Tsokana et al., 2015). This hypothesis is further supported by the exposure of hares to *N. caninum* and *T. gondii* that is indicative of the fact that hares share the same living space with their definitive hosts, that is wild carnivores such as gray wolf and dogs and cats or free-ranging felids respectively. Moreover, as already mentioned, the hare-red fox contact is well documented as hare is a common prey for red foxes (Bakaloudis et al., 2015; Kidawa and Kowalczyk, 2011). The fact that hares exposed to *L. infantum* share the same habitat with these animal species which are susceptible to *L. infantum* infection (Millán et al., 2014; Souza et al., 2014), highlights the need to investigate their role in the sylvatic transmission cycle of *L. infantum* in Greece. This is of further importance when considering animal species such as cats; they live in close proximity to humans while at the same time they are nocturnal predators, adaptive in different kinds of habitats that operate in a 1.5 km radius of their residence using forests as hunting territory (Soares et al., 2016). These feline behavioral characteristics together with the so-called home range of hares, make the cat-to-hare and vice versa spillover of pathogens possible and cats an excellent candidate as a connective link between sylvatic and domestic transmission cycle of *Leishmania* spp. The same holds true for red foxes as they dwell in great distances according to the availability of resources (Bateman and Fleming, 2012), a behavioral characteristic that has enabled red foxes to inhabit suburban and urban areas resulting to close contact with domestic and wild animals and humans.

As a vector-borne disease, the distribution of Leishmaniasis is highly affected by anthropic and climate factors as well as environmental parameters influencing the ecology of vectors and reservoir hosts by altering their activity, survival and abundance (Aspöck et al., 2008; Barón et al., 2011; Elnaiem et al., 1998; Gage et al., 2008; Ozbel et al., 2011). Regarding the vector itself, conflicting results have been obtained by different studies on the influence of such factors on their presence and abundance

depending on the species analyzed (Chamaillé et al., 2010; Gálvez et al., 2010; Martín-Sánchez et al., 2009).

In our study, precipitation was the environmental variable with the highest gain when used in isolation which is in great agreement with a study conducted previously in European Brown hares from Northern Greece (Tsokana et al., 2015). The impact of precipitation on the activity period of sandflies, their survival and the promotion of adult emergence as well as the determination of the appropriate oviposition sites has been previously proposed (Gage et al., 2008; Queiroz et al., 2012; Quintana et al., 2013). Moreover, the impact of precipitation on the increase of food availability supporting high densities of hosts cannot be ruled out.

The land cover was also recognized as an important factor affecting the exposure of hares to *L.infantum* with natural grasslands, coniferous forests, land principally occupied by agriculture, with significant areas of natural vegetation and non-irrigated arable land being the associated categories. Such land types are characterized by moderate to high annual precipitation providing moist and shaded microenvironments, rich in organic matter, thus influencing the sandfly vector as mentioned previously (Gage et al., 2008; Queiroz et al., 2012; Quintana et al., 2013). Moreover, their form supports a vast variety of mammals and diverse wildlife including important hosts of *L.infantum* such as free-ranging cats and rodents thus constituting an excellent space for the overlapping of sylvatic and domestic *L.infantum* transmission cycle. In particular, the agricultural and arable land has been previously related with increased probability of *Leishmania* infection is humans and dogs in our study area (Giannakopoulos et al., 2016), in Greece (Ntais et al., 2013) and in other countries as well (Alonso et al., 2010; Chamaillé et al., 2010; Colacicco-Mayhugh et al., 2010) possibly due to the increased humidity and the host abundance such as rodents, as a result of the increased food availability.

Although temperature, altitude, wind speed and livestock density were recognized as important factors for model development, they were not among the leading factors. The importance of temperature has been repeatedly suggested by previous studies mainly affecting the biology and ecology of sandflies (rate of egg production, development of the juvenile stages, number of annual generations, feeding activity, period of activity and survival of adults) as well as the *Leishmania* spp development

inside the vector (Ballart et al., 2014; Elnaiem et al., 1998; Martín-Sánchez et al., 2009; Ready, 2008). The influence of temperature on the presence and abundance of sandflies is highly dependent of the species analysed or the bioclimatic area considered (Chamaillé et al., 2010; Gálvez et al., 2010; Martín-Sánchez et al., 2009) suggesting that the vector distribution and population density is not solely influenced by temperature itself. Altitude, despite rather being a confounding factor instead of an ecological factor, negatively impacts the distribution of the sandfly species, possibly due to its close correlation with vegetation and meteorological conditions (Elnaiem et al., 1998; Ferreira et al., 2011; Gebre-Michael et al., 2004; Guernaoui et al., 2006; Ozbel et al., 2011). In fact, the increased altitude is linked to changes in vegetation, decrease of the temperature and increase in the precipitation, that is, a more hostile environment for sand fly survival (Gálvez et al., 2010). Wind speed has been also previously suggested as a factor affecting the abundance of sandflies (Ntais et al., 2013) while significantly higher vector densities were detected at sites sheltered from the wind because of the vector preference for more natural, protected sites (Gálvez et al., 2010). Conflicting results have been obtained regarding the correlation of livestock and sanfly density with some studies suggesting that the presence of livestock, especially cattle and chicken, is linked with an increase in vector densities (Alexander et al., 2002; Ferro et al., 1997; Guernaoui and Boumezzough, 2009; Maroli et al., 2009) possibly because the set-up of their breeding sites is favored, while others found no association (Ballart et al., 2014).

## Conclusions

Although a species with limited home range, hares may significantly contribute to the epidemiology of important pathogens of public health and veterinary concern due to cross border and long-distance movements possibly as part of live animal translocations, as the host of vectors and the prey of carnivores and omnivores that travel in great distances and get into contact with domestic animals and humans. Besides, its short life span makes this species an excellent indicator for recent pathogen transmission in the area and its intensive contact with vectors such as ticks, mosquitoes and sandflies has implicated hare in the epidemiology of important vector borne diseases such as Leishmaniasis. The continuous surveillance of hare populations could

provide information on the population health status and the pathogens circulating in the area posing risk for wildlife, domestic animals and humans.

## References


Afonso, E., Germain, E., Poulle, M.-L., Ruette, S., Devillard, S., Say, L., Villena, I., Aubert, D., Gilot-Fromont, E., 2013. Environmental determinants of spatial and temporal variations in the transmission of Toxoplasma gondii in its definitive hosts. Int. J. Parasitol. Parasites Wildl. 2, 278–285. https://doi.org/10.1016/j.ijppaw.2013.09.006

Afonso, E., Thulliez, P., Gilot-Fromont, E., 2010. Local meteorological conditions, dynamics of seroconversion to Toxoplasma gondii in cats ( Felis catus) and oocyst burden in a rural environment. Epidemiol. Infect. 138, 1105–1113. https://doi.org/10.1017/S0950268809991270

Alexander, B., de Carvalho, R.L., McCallum, H., Pereira, M.H., 2002. Role of the domestic chicken (Gallus gallus) in the epidemiology of urban visceral leishmaniasis in Brazil. Emerg. Infect. Dis. 8, 1480–1485. https://doi.org/10.3201/eid0812.010485

Almería, S., Calvete, C., Pagés, A., Gauss, C., Dubey, J.P., 2004. Factors affecting the seroprevalence of Toxoplasma gondii infection in wild rabbits (Oryctolagus cuniculus) from Spain. Vet. Parasitol. 123, 265–270. https://doi.org/10.1016/j.vetpar.2004.06.010

Alonso, F., Giménez Font, P., Manchón, M., Ruiz de Ybáñez, R., Segovia, M., Berriatua, E., 2010. Geographical variation and factors associated to seroprevalence of canine leishmaniosis in an endemic Mediterranean area. Zoonoses Public Health 57, 318–328. https://doi.org/10.1111/j.1863-2378.2008.01228.x

Alves, P.C., Ferrand, N., Hackländer, K. (Eds.), 2008. Lagomorph biology: evolution, ecology, and conservation. Springer, Berlin ; New York.

Diakou, A., Papadopoulos, E., Panousis, N., Karatzias, C., Giadinis, N., 2013. Toxoplasma gondii and Neospora caninum seroprevalence in dairy sheep and goats mixed stock farming. Vet. Parasitol. 198, 387–390. https://doi.org/10.1016/j.vetpar.2013.09.017

Arboleda, S., Jaramillo-O., N., Peterson, A.T., 2009. Mapping Environmental Dimensions of Dengue Fever Transmission Risk in the Aburrá Valley, Colombia. Int. J. Environ. Res. Public. Health 6, 3040–3055. https://doi.org/10.3390/ijerph6123040

Aspöck, H., Gerersdorfer, T., Formayer, H., Walochnik, J., 2008. Sandflies and sandfly-borne infections of humans in Central Europe in the light of climate change. Wien. Klin. Wochenschr. 120, 24–29. https://doi.org/10.1007/s00508-008-1072-8

Athanasiou, L.V., Kontos, V.I., Saridomichelakis, M.N., Rallis, T.S., Diakou, A., 2012. A cross-sectional sero-epidemiological study of canine leishmaniasis in Greek mainland. Acta Trop. 122, 291–295. https://doi.org/10.1016/j.actatropica.2012.02.003

Aubert, D., Ajzenberg, D., Richomme, C., Gilot-Fromont, E., Terrier, M.E., de Gevigney, C., Game, Y., Maillard, D., Gibert, P., Dardé, M.L., Villena, I., 2010. Molecular and biological characteristics of Toxoplasma gondii isolates from wildlife in France. Vet. Parasitol. 171, 346–349. https://doi.org/10.1016/j.vetpar.2010.03.033

Bakaloudis, D.E., Bontzorlos, V.A., Vlachos, C.G., Papakosta, M.A., Chatzinikos, E.N., Braziotis, S.G., Kontsiotis, V.J., 2015. Factors affecting the diet of the red fox (Vulpes vulpes) in a heterogeneousMediterranean landscape. Turk. J. Zool. 39, 1151–1159. https://doi.org/10.3906/zoo-1411-22

Ballart, C., Guerrero, I., Castells, X., Barón, S., Castillejo, S., Alcover, M.M., Portús, M., Gállego, M., 2014. Importance of individual analysis of environmental and climatic factors affecting the density of Leishmania vectors living in the same geographical area: the example of Phlebotomus ariasi and P. perniciosus in northeast Spain. Geospatial Health 8, 389–403. https://doi.org/10.4081/gh.2014.28

Barón, S.D., Morillas-Márquez, F., Morales-Yuste, M., Díaz-Sáez, V., Irigaray, C., Martín-Sánchez, J., 2011. Risk maps for the presence and absence of Phlebotomus perniciosus in an endemic area of leishmaniasis in southern Spain: implications for the control of the disease. Parasitology 138, 1234–1244. https://doi.org/10.1017/S0031182011000953

Bartley, P.M., Wright, S.E., Zimmer, I.A., Roy, S., Kitchener, A.C., Meredith, A., Innes, E.A., Katzer, F., 2013. Detection of Neospora caninum in wild carnivorans in Great Britain. Vet. Parasitol. 192, 279–283. https://doi.org/10.1016/j.vetpar.2012.10.001

Bartova, E., Sedlak, K., 2012. Toxoplasmosis in Animals in the Czech Republic - The Last 10 Years, in: Djurkovi Djakovi, O. (Ed.), Toxoplasmosis - Recent Advances. InTech.

Bártová, E., Sedlák, K., Treml, F., Holko, I., Literák, I., 2010. Neospora caninum and Toxoplasma gondii antibodies in European brown hares in the Czech Republic, Slovakia and Austria. Vet. Parasitol. 171, 155–158. https://doi.org/10.1016/j.vetpar.2010.03.002

Bártová, E., Slezáková, R., Nágl, I., Sedlák, K., 2016. Neospora caninum and Toxoplasma gondii antibodies in red foxes (Vulpes vulpes) in the Czech Republic. Ann. Agric. Environ. Med. AAEM 23, 84–86. https://doi.org/10.5604/12321966.1196858

Bateman, P.W., Fleming, P.A., 2012. Big city life: carnivores in urban environments: Urban carnivores. J. Zool. 287, 1–23. https://doi.org/10.1111/j.1469-7998.2011.00887.x

Bettiol, S.S., Obendorf, D.L., Nowarkowski, M., Milstein, T., Goldsmid, J.M., 2000. Earthworms as paratenic hosts of toxoplasmosis in eastern barred bandicoots in Tasmania. J. Wildl. Dis. 36, 145–148. https://doi.org/10.7589/0090-3558-36.1.145

Catar, G., 1972. Studies on Toxoplasmosis as regards its natural focality in Slovakia. Folia Parasitol. (Praha) 253–256.

Ceccarelli, S., Balsalobre, A., Susevich, M., Echeverria, M., Gorla, D., Marti, G., 2015. Modelling the potential geographic distribution of triatomines infected by Triatoma virus in the southern cone of South America. Parasit. Vectors 8, 153. https://doi.org/10.1186/s13071-015-0761-1

Chamaillé, L., Tran, A., Meunier, A., Bourdoiseau, G., Ready, P., Dedet, J.-P., 2010. Environmental risk mapping of canine leishmaniasis in France. Parasit. Vectors 3, 31. https://doi.org/10.1186/1756-3305-3-31

Colacicco-Mayhugh, M.G., Masuoka, P.M., Grieco, J.P., 2010. Ecological niche model of Phlebotomus alexandri and P. papatasi (Diptera: Psychodidae) in the Middle East. Int. J. Health Geogr. 9, 2. https://doi.org/10.1186/1476-072X-9-2

Diakou, A., Papadopoulos, E., Lazarides, K., 2009. Specific anti-Leishmania spp. antibodies in stray cats in Greece. J. Feline Med. Surg. 11, 728–730. https://doi.org/10.1016/j.jfms.2008.01.009

Du, F., Zhang, Q., Yu, Q., Hu, M., Zhou, Y., Zhao, J., 2012. Soil contamination of Toxoplasma gondii oocysts in pig farms in central China. Vet. Parasitol. 187, 53–56. https://doi.org/10.1016/j.vetpar.2011.12.036

Dubey, J.P., 2010. Toxoplasmosis of animals and humans, 2nd ed. ed. CRC Press, Boca Raton.

Dubey, J.P., Schares, G., 2011. Neosporosis in animals--the last five years. Vet. Parasitol. 180, 90–108. https://doi.org/10.1016/j.vetpar.2011.05.031

Ebani, V.V., Poli, A., Rocchigiani, G., Bertelloni, F., Nardoni, S., Papini, R.A., Mancianti, F., 2016. Serological survey on some pathogens in wild brown hares (Lepus europaeus) in Central Italy. Asian Pac. J. Trop. Med. 9, 465–469. https://doi.org/10.1016/j.apjtm.2016.03.032

Edelhofer, R., Heppe-Winger, E.M., Haßl, A., Aspöck, H., 1989. Toxoplasma infections in game in Eastern Austria. Mitt. Österr. Ges. Tropenmed. Parasitol. 119–123.

El Tai, N.O., El Fari, M., Mauricio, I., Miles, M.A., Oskam, L., El Safi, S.H., Presber, W.H., Schönian, G., 2001. Leishmania donovani: intraspecific polymorphisms of Sudanese isolates revealed by PCR-based analyses and DNA sequencing. Exp. Parasitol. 97, 35–44. https://doi.org/10.1006/expr.2001.4592

Elnaiem, D.A., Connor, S.J., Thomson, M.C., Hassan, M.M., Hassan, H.K., Aboud, M.A., Ashford, R.W., 1998. Environmental determinants of the distribution of Phlebotomus orientalis in Sudan. Ann. Trop. Med. Parasitol. 92, 877–887.

Fernández-Aguilar, X., Alzaga, V., Villanúa, D., Cabezón, O., García-Bocanegra, I., Dubey, J.P., Almería, S., 2013. Epidemiology and prevalence of Toxoplasma gondii infection in the Iberian hare (Lepus granatensis). Vet. Parasitol. 196, 194–198. https://doi.org/10.1016/j.vetpar.2013.01.061

Ferreira, J.P., Leitão, I., Santos-Reis, M., Revilla, E., 2011. Human-Related Factors Regulate the Spatial Ecology of Domestic Cats in Sensitive Areas for

Conservation. PLoS ONE 6, e25970. https://doi.org/10.1371/journal.pone.0025970

Ferro, C., Pardo, R., Torres, M., Morrison, A.C., 1997. Larval microhabitats of Lutzomyia longipalpis (Diptera: Psychodidae) in an endemic focus of visceral leishmaniasis in Colombia. J. Med. Entomol. 34, 719–728.

Ferroglio, E., Trisciuoglio, A., 2003. Antibodies to Neospora caninum in European brown hare (Lepus europaeus). Vet. Parasitol. 115, 75–78. https://doi.org/10.1016/S0304-4017(03)00201-2

Frenkel, J.K., Hassanein, K.M., Hassanein, R.S., Brown, E., Thulliez, P., Quintero-Nunez, R., 1995. Transmission of Toxoplasma gondii in Panama City, Panama: a five-year prospective cohort study of children, cats, rodents, birds, and soil. Am. J. Trop. Med. Hyg. 53, 458–468.

Frölich, K., Wisser, J., Schmüser, H., Fehlberg, U., Neubauer, H., Grunow, R., Nikolaou, K., Priemer, J., Thiede, S., Streich, W.J., Speck, S., 2003. Epizootiologic and ecologic investigations of European brown hares (Lepus europaeus) in selected populations from Schleswig-Holstein, Germany. J. Wildl. Dis. 39, 751–761. https://doi.org/10.7589/0090-3558-39.4.751

Gage, K.L., Burkot, T.R., Eisen, R.J., Hayes, E.B., 2008. Climate and vectorborne diseases. Am. J. Prev. Med. 35, 436–450. https://doi.org/10.1016/j.amepre.2008.08.030

Gálvez, R., Descalzo, M.A., Miró, G., Jiménez, M.I., Martín, O., Dos Santos-Brandao, F., Guerrero, I., Cubero, E., Molina, R., 2010. Seasonal trends and spatial relations between environmental/meteorological factors and leishmaniosis sand fly vector abundances in Central Spain. Acta Trop. 115, 95–102. https://doi.org/10.1016/j.actatropica.2010.02.009

Gamarra, J.A., Cabezón, O., Pabón, M., Arnal, M.C., Luco, D.F., Dubey, J.P., Gortázar, C., Almeria, S., 2008. Prevalence of antibodies against Toxoplasma gondii in roe deer from Spain. Vet. Parasitol. 153, 152–156. https://doi.org/10.1016/j.vetpar.2008.01.028

Gao, X., Wang, Hongbin, Wang, Huan, Qin, H., Xiao, J., 2016. Land use and soil contamination with Toxoplasma gondii oocysts in urban areas. Sci. Total Environ. 568, 1086–1091. https://doi.org/10.1016/j.scitotenv.2016.06.165

Gebre-Michael, T., Malone, J.B., Balkew, M., Ali, A., Berhe, N., Hailu, A., Herzi, A.A., 2004. Mapping the potential distribution of Phlebotomus martini and P. orientalis (Diptera: Psychodidae), vectors of kala-azar in East Africa by use of geographic information systems. Acta Trop. 90, 73–86.

Giannakopoulos, A., Tsokana, C.N., Papadopoulos, E., Spyrou, V., Chatzopoulos, D.C., Valiakos, G., Touloudi, A., Athanasiou, L.V., Billinis, C., 2017. Molecular investigation and geographical distribution of Leishmania spp infection in stray and owned cats (Felis catus) in Thessaly, central Greece. J Hellenic Vet Med Soc 68, 27–34.

Giannakopoulos, A, Tsokana, C.N., Pervanidou, D., Papadopoulos, E., Papaspyropoulos, K., Spyrou, V., Rodi Burriel, A., Vakali, A., Hadjichristodoulou, C., Billinis, C., 2016. Environmental parameters as risk factors for human and canine Leishmania infection in Thessaly, Central Greece. Parasitology 143, 1179–1186. https://doi.org/10.1017/S0031182016000378

Giannakopoulos, A., Valiakos, G., Papaspyropoulos, K., Dougas, G., Korou, L.M., Tasioudi, K.E., Fthenakis, G.C., Hutchings, M.R., Kaimaras, D., Tsokana, C.N., Iliadou, P., Spyrou, V., Tzani, M., Birtsas, P., Kostoglou, P., Sokos, C., Doudounakis, S., Yon, L., Hannant, D., Artois, M., Tsiodras, S., Hadjichristodoulou, C., Billinis, C., 2016. Rabies outbreak in Greece during 2012-2014: use of Geographical Information System for analysis, risk assessment and control. Epidemiol. Infect. 144, 3068–3079. https://doi.org/10.1017/S0950268816001527

Gondim, L.F.P., 2006. Neospora caninum in wildlife. Trends Parasitol. 22, 247–252. https://doi.org/10.1016/j.pt.2006.03.008

Gondim, L.F.P., McAllister, M.M., Pitt, W.C., Zemlicka, D.E., 2004. Coyotes (Canis latrans) are definitive hosts of Neospora caninum. Int. J. Parasitol. 34, 159–161. https://doi.org/10.1016/j.ijpara.2004.01.001

Gotteland, C., Gilot-Fromont, E., Aubert, D., Poulle, M.-L., Dupuis, E., Dardé, M.-L., Forin-Wiart, M.-A., Rabilloud, M., Riche, B., Villena, I., 2014. Spatial distribution of Toxoplasma gondii oocysts in soil in a rural area: Influence of cats and land use. Vet. Parasitol. 205, 629–637. https://doi.org/10.1016/j.vetpar.2014.08.003

Guernaoui, S., Boumezzough, A., 2009. Habitat preferences of phlebotomine sand flies (Diptera: Psychodidae) in southwestern Morocco. J. Med. Entomol. 46, 1187–1194.

Guernaoui, S., Boumezzough, A., Laamrani, A., 2006. Altitudinal structuring of sand flies (Diptera: Psychodidae) in the High-Atlas mountains (Morocco) and its relation to the risk of leishmaniasis transmission. Acta Trop. 97, 346–351. https://doi.org/10.1016/j.actatropica.2006.01.001

Gustafsson, K., Uggla, A., 1994. Serologic survey for Toxoplasma gondii infection in the Brown hare (Lepus Europaeus P.) in Sweden. J. Wildl. Dis. 30, 201–204. https://doi.org/10.7589/0090-3558-30.2.201

Gustafsson, K., Uggla, A., Järplid, B., 1997. Toxoplasma gondii infection in the mountain hare (Lepus timidus) and domestic rabbit (Oryctolagus cuniculus). I. Pathology. J. Comp. Pathol. 117, 351–360.

Hijmans, R.J., Cameron, S.E., Parra, J.L., Jones, P.G., Jarvis, A., 2005. Very high resolution interpolated climate surfaces for global land areas. Int. J. Climatol. 25, 1965–1978. https://doi.org/10.1002/joc.1276

Homan, W.., Vercammen, M., De Braekeleer, J., Verschueren, H., 2000. Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in Toxoplasma gondii, and its use for diagnostic and quantitative PCR1Note: Nucleotide sequence data reported in this paper have been submitted to GenBankTM database with the accession number AF146527 (Toxoplasma gondii genomic repetitive 529 bp fragment).1. Int. J. Parasitol. 30, 69–75. https://doi.org/10.1016/S0020-7519(99)00170-8

Jokelainen, P., 2012. Endemic Toxoplasma gondii Genotype II Causes Fatal Infections in Animal Hosts in Europe - Lessons Learnt, in: Djurkovi Djakovi, O. (Ed.), Toxoplasmosis - Recent Advances. InTech. https://doi.org/10.5772/49984

Jokelainen, P., Isomursu, M., Näreaho, A., Oksanen, A., 2011. Natural toxoplasma gondii infections in European brown hares and mountain hares in Finland: proportional mortality rate, antibody prevalence, and genetic characterization. J. Wildl. Dis. 47, 154–163. https://doi.org/10.7589/0090-3558-47.1.154

Jores, J., Derocher, A.E., Staubach, C., Aschfalk, A., 2008. Occurrence and prevalence of Clostridium perfringens in polar bears from Svalbard, Norway. J. Wildl. Dis. 44, 155–158. https://doi.org/10.7589/0090-3558-44.1.155

Kantzoura, V., Diakou, A., Kouam, M.K., Feidas, H., Theodoropoulou, H., Theodoropoulos, G., 2013. Seroprevalence and risk factors associated with zoonotic parasitic infections in small ruminants in the Greek temperate environment. Parasitol. Int. 62, 554–560. https://doi.org/10.1016/j.parint.2013.08.010

Karayiannis, S., Ntais, P., Messaritakis, I., Tsirigotakis, N., Dokianakis, E., Antoniou, M., 2015. Detection of Leishmania Infantum in red foxes (Vulpes vulpes) in Central Greece. Parasitology 142, 1574–1578. https://doi.org/10.1017/S0031182015001158

Kidawa, D., Kowalczyk, R., 2011. The effects of sex, age, season and habitat on diet of the red fox Vulpes vulpes in northeastern Poland. Acta Theriol. (Warsz.) 56, 209–218. https://doi.org/10.1007/s13364-011-0031-3

King, J.S., Šlapeta, J., Jenkins, D.J., Al-Qassab, S.E., Ellis, J.T., Windsor, P.A., 2010. Australian dingoes are definitive hosts of Neospora caninum. Int. J. Parasitol. 40, 945–950. https://doi.org/10.1016/j.ijpara.2010.01.008

Kitts-Morgan, S.E., Caires, K.C., Bohannon, L.A., Parsons, E.I., Hilburn, K.A., 2015. Free-Ranging Farm Cats: Home Range Size and Predation on a Livestock Unit In Northwest Georgia. PLOS ONE 10, e0120513. https://doi.org/10.1371/journal.pone.0120513

Kouam, M.K., Diakou, A., Kanzoura, V., Papadopoulos, E., Gajadhar, A.A., Theodoropoulos, G., 2010. A seroepidemiological study of exposure to Toxoplasma, Leishmania, Echinococcus and Trichinella in equids in Greece and analysis of risk factors. Vet. Parasitol. 170, 170–175. https://doi.org/10.1016/j.vetpar.2010.02.004

Kunst, P.J.G., van der Wal, R., van Wieren, S., 2001. Home ranges of brown hares in a natural salt marsh: comparisons with agricultural systems. Acta Theriol. (Warsz.) 46, 287–294. https://doi.org/10.1007/BF03192435

Lehmann, T., Graham, D.H., Dahl, E., Sreekumar, C., Launer, F., Corn, J.L., Gamble, H.R., Dubey, J.P., 2003. Transmission dynamics of Toxoplasma gondii on a pig farm. Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis. 3, 135–141.

Leite, R.S., Ferreira, S. de A., Ituassu, L.T., de Melo, M.N., de Andrade, A.S.R., 2010. PCR diagnosis of visceral leishmaniasis in asymptomatic dogs using conjunctival swab samples. Vet. Parasitol. 170, 201–206. https://doi.org/10.1016/j.vetpar.2010.02.020

Liberg, O., 1980. Spacing Patterns in a Population of Rural Free Roaming Domestic Cats. Oikos 35, 336. https://doi.org/10.2307/3544649

Lindsay, D.S., Dubey, J.P., 2014. Toxoplasmosis in Wild and Domestic Animals, in: Toxoplasma Gondii. Elsevier, pp. 193–215.

Lindsay, D.S., Weston, J.L., Little, S.E., 2001. Prevalence of antibodies to Neospora caninum and Toxoplasma gondii in gray foxes (Urocyon cinereoargenteus) from South Carolina. Vet. Parasitol. 97, 159–164.

Maroli, M., Jalouk, L., Al Ahmed, M., Bianchi, R., Bongiorno, G., Khoury, C., Gradoni, L., 2009. Aspects of the bionomics of Phlebotomus sergenti sandflies from an endemic area of anthroponotic cutaneous leishmaniasis in Aleppo Governorate, Syria. Med. Vet. Entomol. 23, 148–154. https://doi.org/10.1111/j.1365-2915.2009.00808.x

Martín-Sánchez, J., Morales-Yuste, M., Acedo-Sánchez, C., Barón, S., Díaz, V., Morillas-Márquez, F., 2009. Canine leishmaniasis in southeastern Spain. Emerg. Infect. Dis. 15, 795–798. https://doi.org/10.3201/eid1505.080969

Millán, J., Ferroglio, E., Solano-Gallego, L., 2014. Role of wildlife in the epidemiology of Leishmania infantum infection in Europe. Parasitol. Res. 113, 2005–2014. https://doi.org/10.1007/s00436-014-3929-2

Miller, M.., Gardner, I.., Kreuder, C., Paradies, D.., Worcester, K.., Jessup, D.., Dodd, E., Harris, M.., Ames, J.., Packham, A.., Conrad, P.., 2002. Coastal freshwater runoff is a risk factor for Toxoplasma gondii infection of southern sea otters ( Enhydra lutris nereis ). Int. J. Parasitol. 32, 997–1006. https://doi.org/10.1016/S0020-7519(02)00069-3

Molina, R., Jiménez, M.I., Cruz, I., Iriso, A., Martín-Martín, I., Sevillano, O., Melero, S., Bernal, J., 2012. The hare (Lepus granatensis) as potential sylvatic reservoir of

Leishmania infantum in Spain. Vet. Parasitol. 190, 268–271. https://doi.org/10.1016/j.vetpar.2012.05.006

Müller, N., Zimmermann, V., Hentrich, B., Gottstein, B., 1996. Diagnosis of Neospora caninum and Toxoplasma gondii infection by PCR and DNA hybridization immunoassay. J. Clin. Microbiol. 34, 2850–2852.

Nakazawa, Y., Emerson, G.L., Carroll, D.S., Zhao, H., Li, Y., Reynolds, M.G., Karem, K.L., Olson, V.A., Lash, R.R., Davidson, W.B., Smith, S.K., Levine, R.S., Regnery, R.L., Sammons, S.A., Frace, M.A., Mutasim, E.M., Karsani, M.E.M., Muntasir, M.O., Babiker, A.A., Opoka, L., Chowdhary, V., Damon, I.K., 2013. Phylogenetic and Ecologic Perspectives of a Monkeypox Outbreak, Southern Sudan, 2005. Emerg. Infect. Dis. 19, 237–245. https://doi.org/10.3201/eid1902.121220

Ntais, P., Sifaki-Pistola, D., Christodoulou, V., Messaritakis, I., Pratlong, F., Poupalos, G., Antoniou, M., 2013. Leishmaniases in Greece. Am. J. Trop. Med. Hyg. 89, 906–915. https://doi.org/10.4269/ajtmh.13-0070

Ozbel, Y., Balcioğlu, I.C., Olgen, M.K., Simsek, F.M., Töz, S.Ö., Ertabaklar, H., Demir, S., Alkan, M.Z., 2011. Spatial distribution of phlebotomine sand flies in the Aydin Mountains and surroundings: the main focus of cutaneous leishmaniasis in western Turkey. J. Vector Ecol. J. Soc. Vector Ecol. 36 Suppl 1, S99–S105. https://doi.org/10.1111/j.1948-7134.2011.00118.x

Papatsiros, V.G., Athanasiou, L.V., Stougiou, D., Papadopoulos, E., Maragkakis, G.G., Katsoulos, P.D., Lefkaditis, M., Kantas, D., Tzika, E.D., Tassis, P.D., Boutsini, S., 2016. Cross-Sectional Serosurvey and Risk Factors Associated with the Presence of Toxoplasma gondii Antibodies in Pigs in Greece. Vector Borne Zoonotic Dis. Larchmt. N 16, 48–53. https://doi.org/10.1089/vbz.2015.1845

Phillips, S.J., Anderson, R.P., Schapire, R.E., 2006. Maximum entropy modeling of species geographic distributions. Ecol. Model. 190, 231–259. https://doi.org/10.1016/j.ecolmodel.2005.03.026

Poli, A., Mancianti, F., Marconcini, A., Cerri, D., Agrimi, P., 1987. Diseases of wild-living hares (Lepus europaeus P.) in Tuscany, in: Diseases of Wild-Living Hares (Lepus Europaeus P.) in Tuscany R. Ippen and H. D. Schroder (Eds.). Presented at the 29th international symposium of diseases of zoo animals, Akademie- Verlag, Berlin, Germany, pp. 341–346.

Queiroz, M.F.M., Varjão, J.R., Moraes, S.C. de, Salcedo, G.E., 2012. Analysis of sandflies (Diptera: Psychodidae) in Barra do Garças, State of Mato Grosso, Brazil, and the influence of environmental variables on the vector density of Lutzomyia longipalpis (Lutz & Neiva, 1912). Rev. Soc. Bras. Med. Trop. 45, 313–317.

Quintana, M., SalomóN, O., Guerra, R., Lizarralde De Grosso, M., Fuenzalida, A., 2013. Phlebotominae of epidemiological importance in cutaneous leishmaniasis in northwestern Argentina: risk maps and ecological niche models. Med. Vet. Entomol. 27, 39–48. https://doi.org/10.1111/j.1365-2915.2012.01033.x

Ready, P.D., 2008. Leishmaniasis emergence and climate change. Rev. Sci. Tech. Int. Off. Epizoot. 27, 399–412.

Ribeiro, L.A., Santos, L.K.N.S.S., Brito, P.A., Maciel, B.M., Da Silva, A.V., Albuquerque, G.R., 2015. Detection of Toxoplasma gondii DNA in Brazilian oysters (Crassostrea rhizophorae). Genet. Mol. Res. GMR 14, 4658–4665. https://doi.org/10.4238/2015.May.4.25

Richomme, C., Afonso, E., Tolon, V., Ducrot, C., Halos, L., Alliot, A., Perret, C., Thomas, M., Boireau, P., Gilot-Fromont, E., 2010. Seroprevalence and factors associated with Toxoplasma gondii infection in wild boar ( Sus scrofa) in a Mediterranean island. Epidemiol. Infect. 138, 1257–1266. https://doi.org/10.1017/S0950268810000117

Rocchigiani, G., Ebani, V.V., Nardoni, S., Bertelloni, F., Bascherini, A., Leoni, A., Mancianti, F., Poli, A., 2018. Molecular survey on the occurrence of arthropod-borne pathogens in wild brown hares ( Lepus europaeus ) from Central Italy. Infect. Genet. Evol. 59, 142–147. https://doi.org/10.1016/j.meegid.2018.02.005

Ruiz-Fons, F., Ferroglio, E., Gortázar, C., 2013. Leishmania infantum in free-ranging hares, Spain, 2004-2010. Euro Surveill. Bull. Eur. Sur Mal. Transm. Eur. Commun. Dis. Bull. 18, 20541.

Simon, J.A., Kurdzielewicz, S., Jeanniot, E., Dupuis, E., Marnef, F., Aubert, D., Villena, I., Poulle, M.-L., 2017. Spatial distribution of soil contaminated with Toxoplasma gondii oocysts in relation to the distribution and use of domestic cat defecation sites on dairy farms. Int. J. Parasitol. 47, 357–367. https://doi.org/10.1016/j.ijpara.2017.01.004

Smallbone, W.A., Chadwick, E.A., Francis, J., Guy, E., Perkins, S.E., Sherrard-Smith, E., Cable, J., 2017. East-West Divide: temperature and land cover drive spatial variation of Toxoplasma gondii infection in Eurasian otters (Lutra lutra) from England and Wales. Parasitology 144, 1433–1440. https://doi.org/10.1017/S0031182017000865

Soares, C.S.A., Duarte, S.C., Sousa, S.R., 2016. What do we know about feline leishmaniosis? J. Feline Med. Surg. 18, 435–442. https://doi.org/10.1177/1098612X15589358

Sotiraki, S., Brozos, C., Samartzi, F., Schares, G., Kiossis, E., Conraths, F.J., 2008. Neospora caninum infection in Greek dairy cattle herds detected by two antibody assays in individual milk samples. Vet. Parasitol. 152, 79–84. https://doi.org/10.1016/j.vetpar.2007.11.016

Souza, T.D., Turchetti, A.P., Fujiwara, R.T., Paixão, T.A., Santos, R.L., 2014. Visceral leishmaniasis in zoo and wildlife. Vet. Parasitol. 200, 233–241. https://doi.org/10.1016/j.vetpar.2013.12.025

Touloudi, A., Valiakos, G., Athanasiou, L.V., Birtsas, P., Giannakopoulos, A., Papaspyropoulos, K., Kalaitzis, C., Sokos, C., Tsokana, C.N., Spyrou, V., Petrovska, L., Billinis, C., 2015. A serosurvey for selected pathogens in Greek European wild boar. Vet. Rec. Open 2, e000077. https://doi.org/10.1136/vetreco-2014-000077

Treml, F., Pikula, J., Bandouchova, H., Horakova, J., 2007. European brown hare as a potential source of zoonotic agents. Vet. Med. (Praha) 52, 451–456.

Tsakmakidis, I., Angelopoulou, K., Dovas, C.I., Dokianakis, E., Tamvakis, A., Symeonidou, I., Antoniou, M., Diakou, A., 2017. Leishmania infection in rodents in Greece. Trop. Med. Int. Health TM IH 22, 1523–1532. https://doi.org/10.1111/tmi.12982

Tsokana, C.N., Sokos, C., Giannakopoulos, A., Mamuris, Z., Birtsas, P., Papaspyropoulos, K., Valiakos, G., Spyrou, V., Lefkaditis, M., Chatzopoulos, D.C., Kantere, M., Manolakou, K., Touloudi, A., Burriel, A.R., Ferroglio, E., Hadjichristodoulou, C., Billinis, C., 2015. First evidence of Leishmania infection in European brown hare (Lepus europaeus) in Greece: GIS analysis and phylogenetic position within the Leishmania spp. Parasitol. Res. https://doi.org/10.1007/s00436-015-4749-8

Tzanidakis, N., Maksimov, P., Conraths, F.J., Kiossis, E., Brozos, C., Sotiraki, S., Schares, G., 2012. Toxoplasma gondii in sheep and goats: Seroprevalence and potential risk factors under dairy husbandry practices. Vet. Parasitol. 190, 340–348. https://doi.org/10.1016/j.vetpar.2012.07.020

Valiakos, G., Papaspyropoulos, K., Giannakopoulos, A., Birtsas, P., Tsiodras, S., Hutchings, M.R., Spyrou, V., Pervanidou, D., Athanasiou, L.V., Papadopoulos, N., Tsokana, C., Baka, A., Manolakou, K., Chatzopoulos, D., Artois, M., Yon, L., Hannant, D., Petrovska, L., Hadjichristodoulou, C., Billinis, C., 2014. Use of wild bird surveillance, human case data and GIS spatial analysis for predicting spatial distributions of West Nile virus in Greece. PloS One 9, e96935. https://doi.org/10.1371/journal.pone.0096935

Valle, D., Tucker Lima, J.M., 2014. Large-scale drivers of malaria and priority areas for prevention and control in the Brazilian Amazon region using a novel multi-pathogen geospatial model. Malar. J. 13, 443. https://doi.org/10.1186/1475-2875-13-443

Wapenaar, W., Jenkins, M.C., O'Handley, R.M., Barkema, H.W., 2006. Neospora caninum-like oocysts observed in feces of free-ranging red foxes (Vulpes vulpes) and coyotes (Canis latrans). J. Parasitol. 92, 1270–1274. https://doi.org/10.1645/GE-913R.1

Yan, C., Liang, L.-J., Zheng, K.-Y., Zhu, X.-Q., 2016. Impact of environmental factors on the emergence, transmission and distribution of Toxoplasma gondii. Parasit. Vectors 9. https://doi.org/10.1186/s13071-016-1432-6

Zanet, S., Chiari, M., Battisti, E., Tizzani, P., Trisciuoglio, A., Taricco, L., Lavazza, A., Ferroglio, E., 2016. Leishmania infantum in wild lagomorphs: An epidemiological survey in Italy, in: Contributions to the 12th Conference of the European Wildlife Disease Association (EWDA), Schumann, A., Wibbelt, G., Greenwood, A.D., Hofer, H., (Eds). Presented at the 12th Conference of the European Wildlife Disease Association (EWDA), Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany, p. 213.

# CHAPTER IV

# PHYLOGENETIC POSITION OF *LEISHMANIA* SPP DETECTED IN HUMANS, DOMESTIC AND WILD ANIMALS IN THESSALY, CENTRAL GREECE

## Abstract

Leishmaniasis is a vector-borne mammalian disease of major human and veterinary medical significance which is endemic in Greece. This study aimed to define the phylogenetic position of *Leishmania* spp detected in humans, domestic and wild animals in Thessaly, central Greece. Clinical samples collected from humans presenting clinical manifestations compatible with leishmaniasis and dogs, cats and hares from Thessaly, central Greece, were examined by Internal Transcribed Spacer-1 (ITS-1) nested PCR for the detection of *Leishmania* DNA. Phylogenetic analysis was performed on *Leishmania* sequences detected in different hosts in conjunction with *Leishmania* sequences retrieved from GenBank. Phylogenetic analysis revealed that the *Leishmania* sequences derived from different hosts in Thessaly, central Greece belong in the *L. donovani* complex. The analysis of ITS1 for microsatellite repeat numbers identified *L. infantum* in all the *Leishmania* sequences included in the study except one, which was identified as *L. donovani* in one feline sample.

Leishmaniasis is highly endemic in Thessaly, central Greece in both canine and human population while the role of other animal hosts, including wildlife, has been recently under investigation. This study provides evidence for the predominance of *L.infantum* circulation in different hosts in Thessaly. Moreover, it is indicative of the possible *L. donovani* infection in a cat from the study area, giving the impetus for further investigation. The integrated surveillance on human, animal and sandfly vector population seems to be a necessity and the intensive typing of natural *Leishmania* infections should become a feature of epidemiological investigations in endemic areas.

## Introduction

Leishmaniases are vector-borne diseases caused by obligate protozoan parasites from the genus *Leishmania* (*Trypanosomatida: Trypanosomatidae*) which are widely distributed across 88 tropical, subtropical and temperate countries, including Greece, with more than 350 million people at risk (Georgiadou et al., 2015a). An estimated 12 million patients suffer from leishmaniasis, with approximately 0.2-0.4 million of new visceral leishmaniasis (VL) and 0.7-1.2 million of new cutaneous leishmaniasis (CL) cases per year worldwide. Overall, infection is caused by more than 20 species of *Leishmania* parasites, which are spread by approximately 30 species of phlebotomine sand flies (Georgiadou et al., 2015a; Oshaghi et al., 2009). Most of the transmission cycles are zoonotic, thus requiring the involvement of reservoir hosts such as domestic and wild animals, and a few being strictly anthroponotic (Quinnell and Courtenay, 2009).

Increased international travel, globalisation, population migration together with the climatic change constitute important factors influencing the spread of *Leishmania* species in new geographical areas where sufficient competent vectors exist under favourable ecological conditions (Antoniou et al., 2013). As the conditions of transmission change and the importation of *Leishmania* species into other geographical regions, including areas previously non-endemic for the disease, is being documented, studies concerning the identification of the *Leishmania* species circulating in different regions become a necessity. The importance of *Leishmania* species identification is further highlighted by the need for proper diagnosis and prognosis of the disease as well as for making decisions regarding treatment and control measures especially in areas with various co-existing *Leishmania* species (SchöNian et al., 2011).

In Greece, an endemic Mediterranean country for VL, the disease burden still carries a remarkable morbidity (Georgiadou et al., 2015b; Gkolfinopoulou et al., 2013). VL is the predominant form, endemic in most islands and coastal regions of the country and CL occurs sporadically (Gkolfinopoulou et al., 2013). *L. infantum* is the responsible species for VL and some cases of CL while anthroponotic CL caused by *L. tropica* is also present in Greece (Gkolfinopoulou et al., 2013; Ready, 2010).

Previous studies conducted in Greece included *L.infantum* strains of human and canine origin from the mainland and islands of the country which were analysed by MLMT genotyping (Gouzelou et al., 2013, 2012; Kuhls et al., 2008). In another study,

*Leishmania* ITS1 sequences were used to infer the phylogenetic relationships among *Leishmania* parasites detected in hares and dogs in Northern Greece and confirmed that they belong in the *L. donovani* complex (Tsokana et al., 2015).

Thessaly, central Greece, is a common tourist destination and an important agricultural area in the country while it includes all the kind of ecosystems that exist in Greece from costal and seaside to alpine ones. An increase in the incidence rate of human leishmaniasis cases in Thessaly in 2012-2014, prompted us previously to investigate the environmental variables related to human leishmaniasis cases and *Leishmania* infection in dogs and to recognize high-risk areas in the region (Giannakopoulos et al., 2016). This study reports the *Leishmania* spp currently circulating in Thessaly, central Greece and aims to characterize the *Leishmania* spp infecting humans, domestic and wild animals in this highly endemic using the sequence analysis of ITS1.

## Materials and Methods

### Compliance with ethical standards

The research protocol concerning the human specimens was approved by the Ethics Committee of the General University Hospital of Larissa (Permit Number: 1530 13/3/2015). A written informed consent was obtained from the patients and the human individuals. The data were analysed anonymously.

The canine and feline samples were collected by private practicing veterinarians for routine diagnostic purposes in the context of the research funding program THALES during the period 2013-2015. No animals were euthanized during the study and efforts taken to ameliorate animal suffering. The study did not involve any experimentation, but was based in samples, that had been collected from the dogs and cats for routine diagnostic purposes. Diagnostic veterinary procedures are not within the context of relevant EU legislation for animal experimentations (Directive 86/609/EC) and may be performed in order to diagnose animal diseases and improve animal welfare.

The hare samples represent material collected opportunistically (no active capture, killing and sampling of wild animals specifically for this study was performed) from animals hunter-harvested by members of the Greek Hunting Federation of Thessaly and Sporades and hunters separately, during the hunting season 2014-2015, according

to the prerequisites of the Greek Legislation (FEK 2154/B'/5-8-2014), and submitted to the Department of Microbiology and Parasitology, Faculty of Veterinary Medicine, University of Thessaly, Karditsa, Greece. Thus, special approval was not necessary and steps to ameliorate suffering were not applicable in this study. Research on animals as defined in the EU Ethics for Researchers document (European Commission, 2007, Ethics for Researchers - Facilitating Research Excellence in FP7, Luxembourg: Office for Official Publications of the European Communities, ISBN 978-92-79-05474-7) is not a part of the study.

### Study area

Thessaly is located in central Greece and has a total area of 14.036 $km^2$, which roughly represents 10.6% of the whole country. Thirty six per cent of the land is flat and 17.1% is semi-mountainous, whereas the remaining 44.9% is mountainous (Domenikiotis et al., 2005). The administrative region of Thessaly consists of four prefectures (Larissa, Trikala, Karditsa and Volos) which include 26 municipalities.

### Human and animal samples

Whole blood samples were collected from 23 patients submitted to the General University Hospital of Larissa, Thessaly when presenting clinical picture compatible with human leishmaniasis. At least one of the following clinical samples, that is, exfoliative epithelial cells using conjunctival swabs, lymph node aspirates and whole blood in EDTA were collected from 223 dogs submitted to veterinary clinics in Thessaly if suspected to be *Leishmania* infected following clinical examination by the private practicing veterinarians. Whole blood in EDTA was collected from 150 clinically healthy cats submitted to veterinary clinics in Thessaly for routine examination. Spleen and liver samples were collected from 79 hunter harvested hares in the region of Thessaly during the hunting season 2014-2015. All the human and animal samples were sent still frozen to the Laboratory of Microbiology and Parasitology, University of Thessaly, Karditsa, Greece and they were stored at –20 °C pending DNA extraction.

## DNA extraction and PCR amplification

Total genomic DNA extraction was performed on the animal and human samples collected using a commercially available DNA extraction kit (Thermo Scientific GeneJET Genomic DNA Purification Kit, Thermo Fisher Scientific, USA) according to the manufacturer's protocol. The purified DNA was stored at -20˚C.

The ITS-1 nested PCR was carried out as described previously (Leite et al., 2010a; Tsokana et al., 2015) using primers LITSR (5′ CTGGATCATTTTCCGATG 3′) and L5.8S (5′ TGATACCACTTATCGCACTT 3′) for the first round and LITSnR (5'-CATTTTCCGATGATTACACC-3') and L5.8Sn (5'-CGTTCTTCAACGAAATAGG-3') for the second round of amplification in all the samples included in the study. The cpb PCR was performed according to Hide and Bañuls, 2006 using the forward and reverse primers 5'-CGTGACGCCGGTGAAGAAT-3' and 5'-CGTGCACTCGGCCGTCTT-3' respectively and K26 gene PCR was carried out using primers K26f (5′-ACGAAGGACTCCGCAAAG-3′) and K26r (5′-TTCCCATCGTTTTGCTG-3′) according to Haralambous et al., 2008 in all the human and hare samples and in 42 canine and 10 feline samples which have been previously found to be *Leishmania* positive by ITS1 PCR. Amplification products were visualized on 2% agarose gel stained with ethidium bromide (0.5 μg/ml).

A PCR was considered positive when a band of the expected size, ∼300 bp for ITS1, 702 bp for the cpb *L. infantum* strains, 741 bp for the cpb *L. donovani* strains, and 284 to approximately 1300 bp was obtained for k26 gene. Two archived bone marrow samples from dogs previously diagnosed with canine leishmaniasis, were used as positive controls for *L.infantum* DNA. The presence of *Leishmania* DNA in the positive control samples has been previously confirmed, using different genus specific PCR assays including minicircle kDNA with primers 13A (5′ GTGGGGGAGGGGCGTTCT 3′) and 13B (5′ATTTTCCACCAACCCCCAGTT 3′) (Reale et al., 1999; Rodgers et al., 1990) and ssu rDNA using primers R221 (5' GGTTCCTTTCCTGATTTACG 3') and R332 (5' GGCCGGTAAAGGCCGAATAG 3') (Meredith et al., 1993). Moreover, the positive control samples were examined with the ITS1 and cpb species-specific PCR assays mentioned above, and the presence of *L.infantum* DNA was confirmed following sequencing of the ITS1 and cpb amplicons.

## Sequencing and sequence analysis

Amplicons from 18 *Leishmania* ITS1 PCR positive samples (four human, five canine, five feline and four hare PCR positive samples), were sequenced. Sequence analysis was performed on a part of the rRNA gene (ITS1 region). The positive PCR products were purified using the PureLink PCR Purification Kit (Invitrogen) and were bidirectionally sequenced using the fluorescent BigDye Terminator Cycle sequencing kit v3.1 (Applied Biosystems), followed by fragment separation with a 3,730xl DNA Analyzer (Applied Biosystems).

Consensus ITS1 sequences were created for the human and animal *Leishmania* sequences of this study and multiple-aligned with reference sequences of *L. donovani*, and *L. infantum* retrieved from GenBank using MEGA 7 software. Phylogenetic analysis was performed and a Maximum parsimony (MP) tree was constructed under the heuristic search option with 100 random-taxon addition replicates and tree bisection–reconnection branch swapping, using PAUP*. Node support was assessed on the basis of 1,000 bootstrap replicates. A Bayesian analysis was also performed with MRBAYES version 3.1 (Huelsenbeck and Ronquist, 2001), under the HKY85 model of sequence evolution. Depending on the data set, random starting trees run for $2\times10^6$ to $8\times10^6$ generations were used, sampled every 100 generations. Burn-in frequency was set to the first 25% of the sampled trees. With some minor differences, the phylogenetic tree based on the Bayesian analysis as well as MP tree showed similar topologies (the tree is available on request).

For species identification of *L. donovani* complex, ITS1 *Leishmania* sequences of this study were multiple-aligned using ClustalW, with ITS1 sequences of previously defined *L. infantum* and *L. donovani*, representing the different types of ITS1 sequences, and manually analyzed for microsatellite repeats (Kuhls et al., 2005).

Table 1. List of database accession numbers and geographical and host origin of the *Leishmania* sequences used in this study

| Strain/ isolate international code | Species | Origin | Host | GenBank accession No. |
|---|---|---|---|---|
| MHOM/FR/95/LPN114 | *L. infantum* | France | Human | AJ634340 |
| MHOM/ES/93/PM1 | *L. infantum* | Spain | Human | AJ634341 |
| MHOM/PT/00/IMT260 | *L. infantum* | Portugal | Human | AJ634344 |
| MHOM/CN/54/Peking | *L. infantum* | China | Human | AJ634345 |
| MHOM/TN/80/IPT1 | *L. infantum* | Tunisia | Human | AJ000289 |
| MHOM/IT/94/ISS1036 | *L. infantum* | Italy | Human | AJ634353 |
| MCAN/FR/87/RM1 | *L. infantum* | France | Canine | AJ634346 |
| MHOM/YE/10/SH2 | *L. infantum* | Yemen | Human | KT751245 |
| MHOM/YE/10/SH15 | *L. infantum* | Yemen | Human | KT751248 |
| MHOM/YE/10/SH27 | *L. infantum* | Yemen | Human | KT751253 |
| MHOM/FR/62/LRC-L47 | *L. infantum* | France | Human | AJ000288.1 |
| GRE 1 human-GRE 4 human | *L. infantum* | Greece | Human (n=4) | KY379083-KY379086 |
| GRE 2 cat – GRE 5 cat | *L. infantum* | Greece | Feline (n=4) | KY379075-KY379078 |
| GRE 8 hare-GRE 11 hare | *L. infantum* | Greece | Hare (n=4) | KY379079-KY379082 |
| GRE 4 dog-GRE 8 dog | *L. infantum* | Greece | Canine (n=5) | KY379070-KY379074 |
| G26 | *L. infantum* | Argentina | Canine | JX448535.1 |
| MHOM/UZ/2007/KOM | *L. infantum* | Uzbekistan | Human | FM164417.1 |
| ITOB/IR/2009/Bilesavar | *L. infantum* | Iran | *Phlebotomus tobbi* | HQ535858.1 |
| ITOB/IR/2012/Savodjbolagh55 | *L. infantum* | Iran | *Phlebotomus tobbi* | KC477100.1 |
| MCAN/UZ/2007/LRC-L1309 | *L. infantum* | Algeria | Canine | FN398341.2 |
| MHOM/BR/2007/JFF_BM | *L. infantum* | Brazil | Human | FN398343.2 |
| GRE 1 cat | *L. donovani* | Greece | Feline (n=1) | KY379069 |
| MHOM/CN/00/Wangjie1 | *L. donovani* | China | Human | AJ000294 |
| MHOM/YE/10/SH3 | *L. donovani* | Yemen | Human | KT751256 |

| | | | | |
|---|---|---|---|---|
| MCAN/SD/00/LEM3946 | *L. donovani* | Sudan | Canine | AJ634356 |
| MHOM/SD/62/LRC-L61 | *L. donovani* | Sudan | Human | AJ634365 |
| MHOM/SD/93/9S | *L. donovani* | Sudan | Human | AJ634372 |
| MHOM/IN/71/LRC-L51a | *L. donovani* | India | Human | AJ000290 |
| MHOM/IN/80/DD8 | *L. donovani* | India | Human | AJ000292 |
| MCAN/MA/2002/AD3 | *L. donovani* | Morocco | Canine | AM901453.1 |
| MHOM/LK/2002/L60b | *L. donovani* | Sri Lanka | Human | AM901448.1 |
| MHOM/IQ/1981/SUKKAR2 | *L. donovani* | Iraq | Human | AM901452.1 |
| LdM | *L. donovani* | Sudan | Human | AJ249616.1 |
| MHOM/ET/2010/DM-607 | *L. donovani* | Ethiopia | Human | FN687759.1 |
| MHOM/BR/2007/JFF | *L. donovani* | Brazil | Human | FN398344.2 |
| MHOM/KE/----/KEN224 | *L. donovani* | Kenya | Human | FN677364.1 |
| MCAN/CN/60/GS1 | *L. donovani* | China | Human | HQ830354.1 |
| Trashigang1 | *L. donovani* | Bhutan | Human | JQ730001.1 |
| MM201521 | *L. donovani complex* | Georgia | Human | KT438681.1 |

## Results

### PCR and sequencing

In total, *Leishmania* DNA was successfully amplified by ITS1 nested PCR in nine human, 85 canine, 20 feline and eight hare samples. Cpb PCR yielded no amplification for any of the samples examined accept 11 canine samples and the positive controls. Similarly, *Leishmania* DNA was not successfully amplified by k26 gene PCR in any of the samples examined except for the positive controls.

In total, 18 amplicons from *Leishmania* ITS1 PCR positive samples (four human, five canine, five feline and four hare samples) were sequenced. The Greek *Leishmania* sequences have been deposited in GenBank under accession numbers KY379069-KY379086 (Table 1) and they were used for the phylogenetic and sequence analysis in this study.

### Phylogenetic and sequence analysis

The phylogenetic analysis performed on 52 *Leishmania* sequences, including four human, five canine, five feline and four hare ITS1 *Leishmania* sequences from Thessaly,

central Greece and reference sequences of *L. donovani* and *L. infantum* retrieved from GenBank, placed all the Greek *Leishmania* sequences derived from different hosts, in the *L. donovani* complex. The homology of the nucleotide sequences between the Greek *Leishmania* sequences was 99.7% for humans and hares, and 99.9% for dogs and cats. The nucleotide homology between the human, canine, feline and hare Greek *Leishmania* sequences was 98.8%, while the nucleotide homology between the Greek *Leishmania* sequences and the sequences belonging to *Leishmania donovani* complex which were retrieved from GenBank, was 99.1%.

Based on the analysis of four polymorphic microsatellites of ITS1 sequences of *L. donovani* complex, including reference sequences representing different types of *L. infantum* and *L. donovani* and the Greek *Leishmania* sequences of this study, one sequence type was identified which was identical to that of *L. infantum* isolates from China and Mediterranean region for all the Greek *Leishmania* sequences except of the "GRE 1 cat" which was identical to that of *L. donovani* (Table 2).

In the maximum parsimony tree based on the ITS-1 sequences (Fig. 1), all the Greek *Leishmania* sequences were clustered with *L.infantum* sequences retrieved from GenBank except for the "GRE 1 cat" which was clustered with the other *L.donovani* sequences retrieved from GenBank with a bootstrap value of 62%.

Table 2. Microsatellite repeat numbers of ITS1 gene found for *L. donovani* complex sequences detected in different hosts in Greece compared to reference strains

| Strain/ isolate international code | Species | Origin | Host | Poly C | Poly A | Poly TA | Poly A | GenBank accession No. | Reference |
|---|---|---|---|---|---|---|---|---|---|
| MHOM/FR/95/LPN114 | *L. infantum* | France | Human | 3 | 6 | 4 | 8 | AJ634340 | (Kuhls et al., 2005) |
| MHOM/ES/93/PM1 | *L. infantum* | Spain | Human | 3 | 6 | 4 | 8 | AJ634341 | (Kuhls et al., 2005) |
| MHOM/PT/00/IMT260 | *L. infantum* | Portugal | Human | 3 | 6 | 4 | 8 | AJ634344 | (Kuhls et al., 2005) |
| MHOM/CN/54/Peking | *L. infantum* | China | Human | 3 | 6 | 4 | 8 | AJ634345 | (Kuhls et al., 2005) |
| MHOM/TN/80/IPT1 | *L. infantum* | Tunisia | Human | 3 | 6 | 4 | 8 | AJ000289 | (Kuhls et al., 2005) |
| MHOM/IT/94/ISS1036 | *L. infantum* | Italy | Human | 3 | 6 | 4 | 8 | AJ634353 | (Kuhls et al., 2005) |
| MCAN/FR/87/RM1 | *L. infantum* | France | Canine | 3 | 6 | 4 | 8 | AJ634346 | (Kuhls et al., 2005) |
| GRE 1 human-GRE 4 human | *L. infantum* | Greece | Human | 3 | 6 | 4 | 8 | KY379083-KY379086 | This study |
| GRE 2 cat – GRE 5 cat | *L. infantum* | Greece | Feline | 3 | 6 | 4 | 8 | KY379075-KY379078 | This study |
| GRE 8 hare-GRE 11 hare | *L. infantum* | Greece | Hare | 3 | 6 | 4 | 8 | KY379079-KY379082 | This study |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| GRE 4 dog-GRE 8 dog | *L. infantum* | Greece | Canine | 3 | 6 | 4 | 8 | KY379070-KY379074 | This study |
| MHOM/FR/62/LRC-L47 | *L. infantum* | France | Human | 3 | 6 | 4 | 8 | AJ000288.1 | (Kuhls et al., 2005) |
| GRE 1 cat | *L. donovani* | Greece | Feline | 3 | 6 | 4 | 7 | KY379069 | This study |
| MHOM/CN/00/Wangjie1 | *L. donovani* | China | Human | 3 | 6 | 4 | 7 | AJ000294 | (Kuhls et al., 2005) |
| MCAN/SD/00/LEM3946 | *L. donovani* | Sudan | Canine | 3 | 6 | 4 | 7 | AJ634356 | (Kuhls et al., 2005) |
| MHOM/SD/62/LRC-L61 | *L. donovani* | Sudan | Human | 2 | 8 | 6 | 8 | AJ634365 | (Kuhls et al., 2005) |
| MHOM/SD/93/9S | *L. donovani* | Sudan | Human | 2 | 9 | 5 | 7 | AJ634372 | (Kuhls et al., 2005) |
| MHOM/IN/71/LRC-L51a | *L. donovani* | India | Human | 2 | 8 | 5 | 7 | AJ000290 | (Kuhls et al., 2005) |
| MHOM/IN/80/DD8 | *L. donovani* | India | Human | 2 | 8 | 5 | 7 | AJ000292 | (Kuhls et al., 2005) |

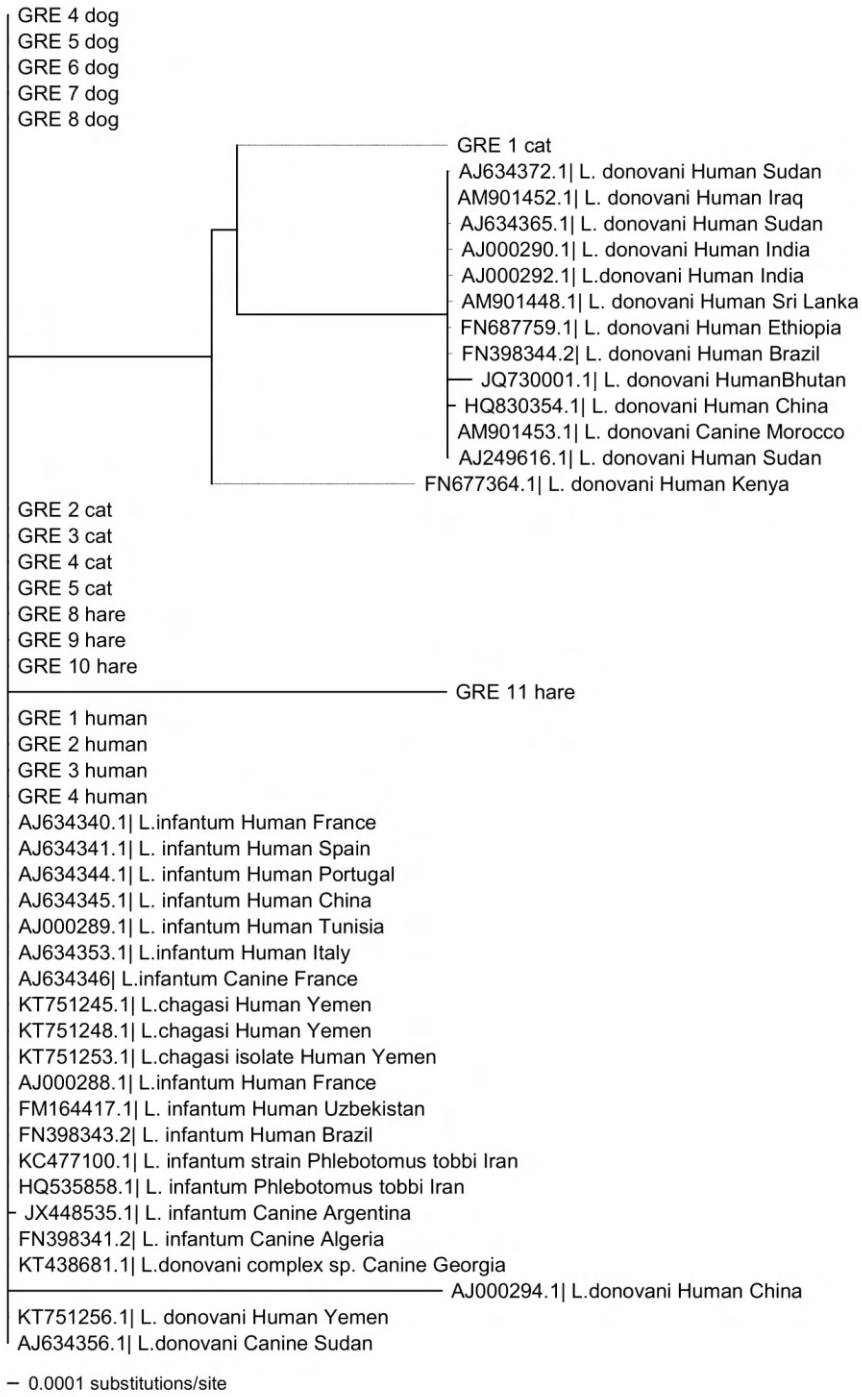


Figure 1. Phylogenetic tree constructed based on ITS1 sequences of 52 *Leishmania* sequences in this study. Phylogenetic tree based on Maximum Parsimony analysis from 52 *Leishmania* sequences (ITS-1 between the genes coding for SSU rRNA and 5.8SrRNA) including the 18 Greek *Leishmania* sequences under study, GRE 1 human-GRE 4 human, GRE 1 cat – GRE 5 cat, GRE 8 hare-GRE 11 hare and GRE 4 dog-GRE 8 dog (accession

numbers KY379069-KY379086). With some minor differences, the phylogenetic tree based on the Bayesian analysis (available on request) showed similar topologies.

## Discussion

In this study, we used *Leishmania* ITS1 sequences derived from *Leishmania* PCR positive humans, domestic and wild animals from Thessaly, central Greece in order to infer the phylogenetic position of the *Leishmania* spp infecting different hosts in the study area. Phylogenetic analysis revealed that the *Leishmania* sequences derived from different hosts in Thessaly belong in the *L. donovani* complex. The analysis of ITS1 for microsatellite repeat numbers identified *L. infantum* in all the *Leishmania* sequences included in the study and *L.donovani* in one feline *Leishmania* sequence.

Differentiation among *Leishmania* spp and in particular between the two representatives of the *L.donovani* complex, the etiological agents of VL, the most severe form of leishmaniasis, is still a subject of study due to the differences in their epidemiology and pathology. Multilocus Enzyme Electrophoresis (MLEE) is still regarded as the reference technique for the identification of *Leishmania* species and subspecies. However, this technique presents important drawbacks (SchöNian et al., 2011). Consequently, several molecular tools have been developed for the identification of the *Leishmania* species based on different coding and non-coding regions in the *Leishmania* genome (SchöNian et al., 2011). PCR-RFLP of the ITS1 is the most widely used assay for direct detection and identification of *Leishmania* species in the Old World (SchöNian et al., 2011). However, representatives of the *L. donovani* complex (*L. donovani* and *L. infantum*) have almost identical RFLP patterns with a great variety of restriction enzymes (SchöNian et al., 2011). On the contrary, sequencing of the 350 bp ITS1 PCR product allows for clear separation of all the medically relevant *Leishmania* species, even *L. infantum* from *L. donovani*, and assigns strains of these species to different phylogenetic groups (Kuhls et al., 2005). Thus, sequence data of the ITS1 region have been used to resolve taxonomic questions and to determine phylogenetic affinities among closely related *Leishmania* species by previous studies as well (Dávila and Momen, 2000; El Tai et al., 2001; Hajjaran et al., 2013; Kuhls et al., 2005; Parvizi et al., 2008; Schönian et al., 2000; Yang et al., 2010). Other PCR assays such as cpb and k26 gene PCR, are discriminative between *L. donovani* and *L. infantum*

species (Haralambous et al., 2008; Hide and Bañuls, 2006). However, both PCR assays require more than 50 pg of DNA for successful amplification (Haralambous et al., 2008; Hide and Bañuls, 2006) and their application may be limited in clinical samples with such a low parasite load as in our case. In particular, in our study, the cpb PCR assay failed to give a detectable PCR product in all the human, hare and feline samples and in 31 out of the 42 canine samples examined while *Leishmania* DNA was successfully amplified by k26 PCR assay only in the canine positive controls. Regarding ITS1 PCR assay, it has been suggested that it is sufficiently sensitive at the level of 0.1 parasites per PCR reaction (Chargui et al., 2012). The fact that in the majority of the samples examined, a positive PCR result was obtained following a second round of amplification (nested ITS1 PCR), is indicative of a quite low parasite load, possibly less than the 50pg of DNA that is required for successful amplification using the cpb and k26 PCR assays. Moreover, these negative results could be possibly attributed to the presence of PCR inhibitors in the human and feline blood samples and in the liver hare samples, but this assumption has not been tested in this study.

*L. infantum* is considered the most widely circulating species in Greece while *L.tropica* causing CL occurs sporadically (Gkolfinopoulou et al., 2013). The results of this study are also suggestive of the predominance of *L. infantum* in humans, dogs, cats and hares in Thessaly, central Greece while *L. tropica* was not detected in any of the samples examined. This finding supports the existence of the zoonotic transmission cycle of *L.infantum* in the study area and is suggestive of the possible overlapping between domestic and sylvatic transmission cycles. Although an adequate number of samples were collected and examined from the different host species, as revealed by the phylogenetic analysis, the genetic variability of *L. infantum* ITS1 microsatellite repeats in circulating strains was rather low. This finding was not surprising; as it has been previously reported, there are almost no differences in the ITS sequences within *L. infantum* strains whereas *L. donovani* strains are more heterogeneous (Kuhls et al., 2005). The use of other molecular markers with greater level of resolution would enable the exploration of the differences between these closely related sequences and would provide differentiation between *Leishmania* parasites at the strain level. However, the kind of the available clinical samples did not make possible the application of such molecular markers. These molecular markers can be used in PCR

fingerprinting, RAPD techniques and PCR-RFLP approaches based on sequence polymorphisms in coding and non-coding regions of multigene families. However, these techniques present several drawbacks in terms of reproducibility and comparability between laboratories (SchöNian et al., 2011). MLEE, multilocus sequence typing (MLST) and multilocus microsatellite typing (MLMT) are the most discriminative ones at intra-species level, allowing further characterization of the parasite diversity, with the latter being the best choice for strain level differentiation due to its better reproducibility and possibility of data storage and exchange (SchöNian et al., 2011). Although the differentiation at strain level was out of the scope of this study, such an analysis would be greatly informative, and we consider this as a limitation of our study. Although *L. donovani* was considered to be limited to East Africa and South Asia, its geographical distribution is gradually expanding with reports coming also from the Mediterranean region and Middle East (Al-Salem et al., 2016; Oshaghi et al., 2009). Apart from the anthroponotic transmission of VL caused by *L. donovani* in East Africa, Bangladesh, India, and Nepal (Al-Salem et al., 2016; Dereure et al., 2003), a zoonotic transmission involving dogs and rodents in East Africa (Dereure et al., 2003; Kassahun et al., 2015), goats in India (Singh et al., 2013) and dogs in Bangladesh (Alam et al., 2013) has been suggested. So far, *L. donovani* has not been identified in any animal host in Greece and no human VL cases caused by *L. donovani* have been reported (Gkolfinopoulou et al., 2013). However, under-reporting or lack of species identification in all the human and animal cases cannot be excluded. The species identification is not regularly performed in Greece in human leishmaniasis cases and in dogs presenting leishmaniasis while the latter are not officially notified despite the law requirements.

To the best of our knowledge, *L. donovani* has not been described in cats previously. Unexpectedly, in this study, *L. donovani* infection was detected in a 12-year-old, male, DSH cat which was admitted to a local veterinary clinic in Karditsa, Thessaly, central Greece for routine examination. At the time of presentation, the owner reported that the cat was living indoors and treatment for endoparasites and vaccinations were performed regularly. Physical examination was normal. The feline samples included in the study were collected in the context of the research program THALES for the epidemiological investigation of *Bartonella* infection in randomly selected clinically healthy cats admitted to veterinary clinics in Thessaly. These samples were

retrospectively examined for *Leishmania* infection and as the initial aim of the study did not involve the investigation of *Leishmania* infection in cats, no collection of further clinical/anamnestic details and data on the disease history of cats was foreseen at the time of the research program implementation. Taken into consideration the initial aim of the study, other biological samples than whole blood in EDTA, were not collected from the cats included in the study. Regarding the sensitivity of PCR assays for *Leishmania* DNA detection, it has been shown that they may be less, equally or even more sensitive when using blood instead of other biological samples (Reithinger et al., 2000; Lachaud et al., 2001; Strauss-Ayali et al., 2004; Manna et al., 2004; Francino et al., 2006; Ferreira et al., 2008; Manna et al., 2008; Leite et al., 2010; de Almeida Ferreira et al., 2012; Lombardo et al., 2012). However, it has been shown that bone marrow, lymph node, spleen, skin and conjunctiva are the tissues presenting the highest sensitivity for the molecular diagnosis of *Leishmania* spp infection (Tsokana et al., 2014). In fact, it has been recently shown that PCR positivity in different tissues from cats ranged from 3.1% (conjunctival swab) to 16% (bone marrow), 18.2% (skin biopsy) and 13% (blood) in clinically normal cats and in cats with various clinical signs living in the region of Thessaly (central Greece) and Thessaloniki (northern Greece), indicating the need for multiple tissue PCR testing to draw solid conclusions on the infection status of a cat (Chatzis et al., 2014). Thus, *L.donovani* DNA could have been probably successfully amplified by the other PCR assays used (cpb and k26 PCR) in this study, if other biological specimens were available from the examined cat. The lack of further clinical/anamnestic details on the *L.donovani* infected cat as well as the absence of other biological samples that did not enable the confirmation of this finding with an alternative diagnostic method or target gene, constitute the major limitations of this study. Consequently, the detection of *L.donovani* infection in this cat, although indicated from our findings, should be treated with caution and should be further investigated in future studies.

In a previous study in the region of Thessaly it was found that 13.3% of the cats examined by ITS1 nested PCR were positive for *Leishmania* DNA supporting the hypothesis that cats may also serve as a reservoir for *Leishmania* spp in this region (Giannakopoulos et al., 2017). In Greece, cats live in close proximity to humans. In the absence of official records, an assessment of the current feline population in Greece is

difficult. However, it appears that stray cats outnumber the owned ones and their population depends on the human population density. Epidemiological studies on the Greek feline population are lacking and unlike stray dogs, no feline management programs exist in Greece; some efforts on stray cats' neutralization are made sporadically at the initiative of local animal welfare associations. The importance of this species stems also for the fact that cats are nocturnal predators, operating in a 1.5 km radius of their residence using forests as hunting territory and they are adaptive in different kinds of habitats. These behavioral characteristics make them vulnerable to exposure and an excellent candidate as a connective link between sylvatic and domestic transmission cycle of *Leishmania* spp (Soares et al., 2016). Besides, the *Leishmania* species that have been identified in cats worldwide are of zoonotic concern (Soares et al., 2016). The present study creates concern on the role of cats in the epidemiology of Leishmaniasis in Greece suggesting that surveillance, design and implementation of management programs on the Greek feline population are crucial.

Regarding the sandfly species that have been reported in the study area, *Phlebotomus perfiliewi*, *P. tobbi*, and *P.neglectus* are considered to be competent vectors of *L.infantum* and *P. similis* is regarded as a competent vector of *L.tropica* (Ivović et al., 2007; Ntais et al., 2013). Additionally, the presence of *P. perfiliewi* in the region of Thessaly (Ntais et al., 2013) seems to be of interest as *P. perfiliewi transcaucasicus*, was previously shown to circulate *L. donovani*, *L. infantum* (Oshaghi et al., 2009) and *L. tropica* (Parvizi et al., 2008) suggesting that this species is a permissive vector. *P. tobbi* could be incriminated for the transmission of both *L. infantum* and *L. donovani*; *L. donovani* was detected in *P. tobbi* in Syria while its feeding preference on humans was recently shown through blood meal identification in Turkey (Mazeris et al., 2010; Svobodová et al., 2009). Thus, the conduction of further entomological studies is highly needed to give insight into the potential vectors of additional *Leishmania spp* in the region and the vector abundance.

The geopolitical position of the country, located at the crossroads of Europe, Asia and Africa, makes Greece a common tourist destination with the vast majority of tourists being from the European Union (EU), followed by those from the Americas, Asia, Oceania and Africa. The last years many immigrants from countries outside the EU moved and settled in Greece. Recently, Greece also became the entrance point of

refugees moving to North Europe. However, the current political circumstances led to the remain and “entrapment” of a great number of refugees in Greece under crowded and poor sanitation conditions. In fact, three refugees’ hosting units are currently operating in the study area. The above-mentioned facts along with the presence of competent vectors, the environmental changes, urbanization and extended agricultural projects seem to make up important risk factors for the circulation of non-endemic *Leishmania* species in Greece.

## Conclusions

The lessons learned from the emergence of new *Leishmania* spp in other countries following the movement of refugees such as from Syria to the neighboring Turkey (Koltas et al., 2014) should give the impetus for integrated and targeted surveillance on human, animal and sandfly vector population in Greece. The conduction of extensive entomological studies is of paramount importance while intensive typing of natural *Leishmania* infections even with PCR assays such as ITS1 PCR followed by RFLP or sequencing analysis, should become a feature of epidemiological investigations in Greece.

## References

Alam, M.Z., Yasin, G., Kato, H., Sakurai, T., Katakura, K., 2013. PCR-based detection of Leishmania donovani DNA in a stray dog from a visceral Leishmaniasis endemic focus in Bangladesh. J. Vet. Med. Sci. 75, 75–78.

Al-Salem, W., Herricks, J.R., Hotez, P.J., 2016. A review of visceral leishmaniasis during the conflict in South Sudan and the consequences for East African countries. Parasit. Vectors 9. https://doi.org/10.1186/s13071-016-1743-7

Alves, P.C., Ferrand, N., Hackländer, K. (Eds.), 2008. Lagomorph biology: evolution, ecology, and conservation. Springer, Berlin ; New York.

Antoniou, M., Gramiccia, M., Molina, R., Dvorak, V., Volf, P., 2013. The role of indigenous phlebotomine sandflies and mammals in the spreading of leishmaniasis agents in the Mediterranean region. Euro Surveill. Bull. Eur. Sur Mal. Transm. Eur. Commun. Dis. Bull. 18, 20540.

Athanasiou, L.V., Kontos, V.I., Saridomichelakis, M.N., Rallis, T.S., Diakou, A., 2012. A cross-sectional sero-epidemiological study of canine leishmaniasis in Greek mainland. Acta Trop. 122, 291–295. https://doi.org/10.1016/j.actatropica.2012.02.003

Chargui, N., Haouas, N., Jaouadi, K., Gorcii, M., Pratlong, F., Dedet, J.P., Mezhoud, H., Babba, H., 2012. Usefulness of a PCR-based method in the detection and species identification of Leishmania from clinical samples. Pathol. Biol. 60, e75–e79. https://doi.org/10.1016/j.patbio.2011.11.011

Chatzis, M.K., Andreadou, M., Leontides, L., Kasabalis, D., Mylonakis, M., Koutinas, A.F., Rallis, T., Ikonomopoulos, J., Saridomichelakis, M.N., 2014. Cytological and molecular detection of Leishmania infantum in different tissues of clinically normal and sick cats. Vet. Parasitol. 202, 217–225. https://doi.org/10.1016/j.vetpar.2014.02.044

Dávila, A.M., Momen, H., 2000. Internal-transcribed-spacer (ITS) sequences used to explore phylogenetic relationships within Leishmania. Ann. Trop. Med. Parasitol. 94, 651–654.

de Almeida Ferreira, S., Leite, R.S., Ituassu, L.T., Almeida, G.G., Souza, D.M., Fujiwara, R.T., de Andrade, A.S.R., Melo, M.N., 2012. Canine skin and conjunctival swab samples for the detection and quantification of Leishmania infantum DNA in an endemic urban area in Brazil. PLoS Negl. Trop. Dis. 6, e1596. https://doi.org/10.1371/journal.pntd.0001596

Dereure, J., El-Safi, S.H., Bucheton, B., Boni, M., Kheir, M.M., Davoust, B., Pratlong, F., Feugier, E., Lambert, M., Dessein, A., Dedet, J.P., 2003. Visceral leishmaniasis in eastern Sudan: parasite identification in humans and dogs; host-parasite relationships. Microbes Infect. 5, 1103–1108.

Domenikiotis, C., Spiliotopoulos, M., Tsiros, E., Dalezios, N.R., 2005. Remotely sensed estimation of annual cotton production under different environmental conditions in Central Greece. Phys. Chem. Earth Parts ABC 30, 45–52. https://doi.org/10.1016/j.pce.2004.08.019

El Tai, N.O., El Fari, M., Mauricio, I., Miles, M.A., Oskam, L., El Safi, S.H., Presber, W.H., Schönian, G., 2001. Leishmania donovani: intraspecific polymorphisms of

Sudanese isolates revealed by PCR-based analyses and DNA sequencing. Exp. Parasitol. 97, 35–44. https://doi.org/10.1006/expr.2001.4592

Ferreira, S. de A., Ituassu, L.T., de Melo, M.N., de Andrade, A.S.R., 2008. Evaluation of the conjunctival swab for canine visceral leishmaniasis diagnosis by PCR-hybridization in Minas Gerais State, Brazil. Vet. Parasitol. 152, 257–263. https://doi.org/10.1016/j.vetpar.2007.12.022

Ferroglio, E., Trisciuoglio, A., 2003. Antibodies to Neospora caninum in European brown hare (Lepus europaeus). Vet. Parasitol. 115, 75–78. https://doi.org/10.1016/S0304-4017(03)00201-2

Francino, O., Altet, L., Sánchez-Robert, E., Rodriguez, A., Solano-Gallego, L., Alberola, J., Ferrer, L., Sánchez, A., Roura, X., 2006. Advantages of real-time PCR assay for diagnosis and monitoring of canine leishmaniosis. Vet. Parasitol. 137, 214–221. https://doi.org/10.1016/j.vetpar.2006.01.011

Georgiadou, S.P., Makaritsis, K.P., Dalekos, G.N., 2015a. Leishmaniasis revisited: Current aspects on epidemiology, diagnosis and treatment. J. Transl. Intern. Med. 3. https://doi.org/10.1515/jtim-2015-0002

Georgiadou, S.P., Stefos, A., Spanakos, G., Skrimpas, S., Makaritsis, K., Sipsas, N.V., Dalekos, G.N., 2015b. Current clinical, laboratory, and treatment outcome characteristics of visceral leishmaniasis: results from a seven-year retrospective study in Greece. Int. J. Infect. Dis. IJID Off. Publ. Int. Soc. Infect. Dis. 34, 46–50. https://doi.org/10.1016/j.ijid.2015.02.021

Giannakopoulos, A., Tsokana, C.N., Papadopoulos, E., Spyrou, V., Chatzopoulos, D.C., Valiakos, G., Touloudi, A., Athanasiou, L.V., Billinis, C., 2017. Molecular investigation and geographical distribution of Leishmania spp infection in stray and owned cats (Felis catus) in Thessaly, central Greece. J Hellenic Vet Med Soc 68, 27–34.

Giannakopoulos, A., Tsokana, C.N., Pervanidou, D., Papadopoulos, E., Papaspyropoulos, K., Spyrou, V., Rodi Burriel, A., Vakali, A., Hadjichristodoulou, C., Billinis, C., 2016. Environmental parameters as risk factors for human and canine Leishmania infection in Thessaly, Central Greece. Parasitology 143, 1179–1186. https://doi.org/10.1017/S0031182016000378

Gkolfinopoulou, K., Bitsolas, N., Patrinos, S., Veneti, L., Marka, A., Dougas, G., Pervanidou, D., Detsis, M., Triantafillou, E., Georgakopoulou, T., Billinis, C., Kremastinou, J., Hadjichristodoulou, C., 2013. Epidemiology of human leishmaniasis in Greece, 1981-2011. Euro Surveill. Bull. Eur. Sur Mal. Transm. Eur. Commun. Dis. Bull. 18, 20532.

Gouzelou, E., Haralambous, C., Amro, A., Mentis, A., Pratlong, F., Dedet, J.-P., Votypka, J., Volf, P., Ozensoy Toz, S., Kuhls, K., Schönian, G., Soteriadou, K., 2012. Multilocus Microsatellite Typing (MLMT) of Strains from Turkey and Cyprus Reveals a Novel Monophyletic L. donovani Sensu Lato Group. PLoS Negl. Trop. Dis. 6, e1507. https://doi.org/10.1371/journal.pntd.0001507

Gouzelou, E., Haralambous, C., Antoniou, M., Christodoulou, V., Martinković, F., Živičnjak, T., Smirlis, D., Pratlong, F., Dedet, J.-P., Özbel, Y., Toz, S.Ö., Presber, W., Schönian, G., Soteriadou, K., 2013. Genetic diversity and structure in Leishmania infantum populations from southeastern Europe revealed by microsatellite analysis. Parasit. Vectors 6, 342. https://doi.org/10.1186/1756-3305-6-342

Hajjaran, H., Mohebali, M., Mamishi, S., Vasigheh, F., Oshaghi, M.A., Naddaf, S.R., Teimouri, A., Edrissian, G.H., Zarei, Z., 2013. Molecular identification and polymorphism determination of cutaneous and visceral leishmaniasis agents isolated from human and animal hosts in Iran. BioMed Res. Int. 2013, 789326. https://doi.org/10.1155/2013/789326

Haralambous, C., Antoniou, M., Pratlong, F., Dedet, J.-P., Soteriadou, K., 2008. Development of a molecular assay specific for the Leishmania donovani complex that discriminates L. donovani/Leishmania infantum zymodemes: a useful tool for typing MON-1. Diagn. Microbiol. Infect. Dis. 60, 33–42. https://doi.org/10.1016/j.diagmicrobio.2007.07.019

Hide, M., Bañuls, A.-L., 2006. Species-specific PCR assay for L. infantum/L. donovani discrimination. Acta Trop. 100, 241–245. https://doi.org/10.1016/j.actatropica.2006.10.012

Huelsenbeck, J.P., Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinforma. Oxf. Engl. 17, 754–755.

Ivović, V., Patakakis, M., Tselentis, Y., Chaniotis, B., 2007. Faunistic study of sandflies in Greece. Med. Vet. Entomol. 21, 121–124. https://doi.org/10.1111/j.1365-2915.2006.00649.x

Karayiannis, S., Ntais, P., Messaritakis, I., Tsirigotakis, N., Dokianakis, E., Antoniou, M., 2015. Detection of Leishmania Infantum in red foxes (Vulpes vulpes) in Central Greece. Parasitology 142, 1574–1578. https://doi.org/10.1017/S0031182015001158

Kassahun, A., Sadlova, J., Dvorak, V., Kostalova, T., Rohousova, I., Frynta, D., Aghova, T., Yasur-Landau, D., Lemma, W., Hailu, A., Baneth, G., Warburg, A., Volf, P., Votypka, J., 2015. Detection of Leishmania donovani and L. tropica in Ethiopian wild rodents. Acta Trop. 145, 39–44. https://doi.org/10.1016/j.actatropica.2015.02.006

Kidawa, D., Kowalczyk, R., 2011. The effects of sex, age, season and habitat on diet of the red fox Vulpes vulpes in northeastern Poland. Acta Theriol. (Warsz.) 56, 209–218. https://doi.org/10.1007/s13364-011-0031-3

Koltas, I.S., Eroglu, F., Alabaz, D., Uzun, S., 2014. The emergence of Leishmania major and Leishmania donovani in southern Turkey. Trans. R. Soc. Trop. Med. Hyg. 108, 154–158. https://doi.org/10.1093/trstmh/trt119

Kuhls, K., Chicharro, C., Cañavate, C., Cortes, S., Campino, L., Haralambous, C., Soteriadou, K., Pratlong, F., Dedet, J.-P., Mauricio, I., Miles, M., Schaar, M., Ochsenreither, S., Radtke, O.A., Schönian, G., 2008. Differentiation and Gene Flow among European Populations of Leishmania infantum MON-1. PLoS Negl. Trop. Dis. 2, e261. https://doi.org/10.1371/journal.pntd.0000261

Kuhls, K., Mauricio, I.L., Pratlong, F., Presber, W., Schönian, G., 2005. Analysis of ribosomal DNA internal transcribed spacer sequences of the Leishmania donovani complex. Microbes Infect. Inst. Pasteur 7, 1224–1234. https://doi.org/10.1016/j.micinf.2005.04.009

Lachaud, L., Chabbert, E., Dubessay, P., Reynes, J., Lamothe, J., Bastien, P., 2001. Comparison of various sample preparation methods for PCR diagnosis of visceral leishmaniasis using peripheral blood. J. Clin. Microbiol. 39, 613–617. https://doi.org/10.1128/JCM.39.2.613-617.2001

Leite, R.S., Ferreira, S. de A., Ituassu, L.T., de Melo, M.N., de Andrade, A.S.R., 2010b. PCR diagnosis of visceral leishmaniasis in asymptomatic dogs using conjunctival swab samples. Vet. Parasitol. 170, 201–206. https://doi.org/10.1016/j.vetpar.2010.02.020

Leontides, L.S., Saridomichelakis, M.N., Billinis, C., Kontos, V., Koutinas, A.F., Galatos, A.D., Mylonakis, M.E., 2002. A cross-sectional study of Leishmania spp. infection in clinically healthy dogs with polymerase chain reaction and serology in Greece. Vet. Parasitol. 109, 19–27.

Lombardo, G., Pennisi, M.G., Lupo, T., Migliazzo, A., Caprì, A., Solano-Gallego, L., 2012. Detection of Leishmania infantum DNA by real-time PCR in canine oral and conjunctival swabs and comparison with other diagnostic techniques. Vet. Parasitol. 184, 10–17. https://doi.org/10.1016/j.vetpar.2011.08.010

Manna, L., Gravino, A.E., Picillo, E., Decaro, N., Buonavoglia, C., 2008. Leishmania DNA quantification by real-time PCR in naturally infected dogs treated with miltefosine. Ann. N. Y. Acad. Sci. 1149, 358–360. https://doi.org/10.1196/annals.1428.018

Manna, L., Vitale, F., Reale, S., Caracappa, S., Pavone, L.M., Morte, R.D., Cringoli, G., Staiano, N., Gravino, A.E., 2004. Comparison of different tissue sampling for PCR-based diagnosis and follow-up of canine visceral leishmaniosis. Vet. Parasitol. 125, 251–262. https://doi.org/10.1016/j.vetpar.2004.07.019

Mazeris, A., Soteriadou, K., Dedet, J.P., Haralambous, C., Tsatsaris, A., Moschandreas, J., Messaritakis, I., Christodoulou, V., Papadopoulos, B., Ivovic, V., Pratlong, F., Loucaides, F., Antoniou, M., 2010. Leishmaniases and the Cyprus Paradox. Am. J. Trop. Med. Hyg. 82, 441–448. https://doi.org/10.4269/ajtmh.2010.09-0282

Meredith, S.E., Zijlstra, E.E., Schoone, G.J., Kroon, C.C., van Eys, G.J., Schaeffer, K.U., el-Hassan, A.M., Lawyer, P.G., 1993. Development and application of the polymerase chain reaction for the detection and identification of Leishmania parasites in clinical material. Arch. Inst. Pasteur Tunis 70, 419–431.

Millán, J., Ferroglio, E., Solano-Gallego, L., 2014. Role of wildlife in the epidemiology of Leishmania infantum infection in Europe. Parasitol. Res. 113, 2005–2014. https://doi.org/10.1007/s00436-014-3929-2

Tsokana, C.N., Athanasiou L.V., Valiakos, G., Spyrou, V., Manolakou, K., Billinis, C., 2014. Molecular Diagnosis of Leishmaniasis, Species Identification and Phylogenetic Analysis, in: Claborn, D. (Ed.), Leishmaniasis - Trends in Epidemiology, Diagnosis and Treatment. InTech.

Ntais, P., Sifaki-Pistola, D., Christodoulou, V., Messaritakis, I., Pratlong, F., Poupalos, G., Antoniou, M., 2013. Leishmaniases in Greece. Am. J. Trop. Med. Hyg. 89, 906–915. https://doi.org/10.4269/ajtmh.13-0070

Oshaghi, M.A., Ravasan, N.M., Hide, M., Javadian, E.-A., Rassi, Y., Sadraei, J., Mohebali, M., Mehdi Sedaghat, M., Hajjaran, H., Zarei, Z., Mohtarami, F., 2009. Phlebotomus perfiliewi transcaucasicus is circulating both Leishmania donovani and L. infantum in northwest Iran. Exp. Parasitol. 123, 218–225. https://doi.org/10.1016/j.exppara.2009.07.004

Parvizi, P., Moradi, G., Akbari, G., Farahmand, M., Ready, P.D., Piazak, N., Assmar, M., Amirkhani, A., 2008. PCR detection and sequencing of parasite ITS-rDNA gene from reservoirs host of zoonotic cutaneous leishmaniasis in central Iran. Parasitol. Res. 103, 1273–1278. https://doi.org/10.1007/s00436-008-1124-z

Quinnell, R.J., Courtenay, O., 2009. Transmission, reservoir hosts and control of zoonotic visceral leishmaniasis. Parasitology 136, 1915–1934. https://doi.org/10.1017/S0031182009991156

Ready, P.D., 2010. Leishmaniasis emergence in Europe. Euro Surveill. Bull. Eur. Sur Mal. Transm. Eur. Commun. Dis. Bull. 15, 19505.

Reale, S., Maxia, L., Vitale, F., Glorioso, N.S., Caracappa, S., Vesco, G., 1999. Detection of Leishmania infantum in dogs by PCR with lymph node aspirates and blood. J. Clin. Microbiol. 37, 2931–2935.

Reithinger, R., Lambson, B.E., Barker, D.C., Davies, C.R., 2000. Use of PCR to detect Leishmania (Viannia) spp. in dog blood and bone marrow. J. Clin. Microbiol. 38, 748–751.

Rodgers, M.R., Popper, S.J., Wirth, D.F., 1990. Amplification of kinetoplast DNA as a tool in the detection and diagnosis of Leishmania. Exp. Parasitol. 71, 267–275.

Schönian, G., Akuffo, H., Lewin, S., Maasho, K., Nylén, S., Pratlong, F., Eisenberger, C.L., Schnur, L.F., Presber, W., 2000. Genetic variability within the species

Leishmania aethiopica does not correlate with clinical variations of cutaneous leishmaniasis. Mol. Biochem. Parasitol. 106, 239–248.

SchöNian, G., Kuhls, K., Mauricio, I.L., 2011. Molecular approaches for a better understanding of the epidemiology and population genetics of Leishmania. Parasitology 138, 405–425. https://doi.org/10.1017/S0031182010001538

Singh, N., Mishra, J., Singh, R., Singh, S., 2013. Animal Reservoirs of Visceral Leishmaniasis in India. J. Parasitol. 99, 64–67. https://doi.org/10.1645/GE-3085.1

Soares, C.S.A., Duarte, S.C., Sousa, S.R., 2016. What do we know about feline leishmaniosis? J. Feline Med. Surg. 18, 435–442. https://doi.org/10.1177/1098612X15589358

Souza, T.D., Turchetti, A.P., Fujiwara, R.T., Paixão, T.A., Santos, R.L., 2014. Visceral leishmaniasis in zoo and wildlife. Vet. Parasitol. 200, 233–241. https://doi.org/10.1016/j.vetpar.2013.12.025

Strauss-Ayali, D., Jaffe, C.L., Burshtain, O., Gonen, L., Baneth, G., 2004. Polymerase chain reaction using noninvasively obtained samples, for the detection of Leishmania infantum DNA in dogs. J. Infect. Dis. 189, 1729–1733. https://doi.org/10.1086/383281

Svobodová, M., Alten, B., Zídková, L., Dvořák, V., Hlavačková, J., Myšková, J., Šeblová, V., Kasap, O.E., Belen, A., Votýpka, J., Volf, P., 2009. Cutaneous leishmaniasis caused by Leishmania infantum transmitted by Phlebotomus tobbi. Int. J. Parasitol. 39, 251–256. https://doi.org/10.1016/j.ijpara.2008.06.016

Tsakmakidis, I., Angelopoulou, K., Dovas, C.I., Dokianakis, E., Tamvakis, A., Symeonidou, I., Antoniou, M., Diakou, A., 2017. Leishmania infection in rodents in Greece. Trop. Med. Int. Health TM IH 22, 1523–1532. https://doi.org/10.1111/tmi.12982

Tsokana, C.N., Sokos, C., Giannakopoulos, A., Mamuris, Z., Birtsas, P., Papaspyropoulos, K., Valiakos, G., Spyrou, V., Lefkaditis, M., Chatzopoulos, D.C., Kantere, M., Manolakou, K., Touloudi, A., Burriel, A.R., Ferroglio, E., Hadjichristodoulou, C., Billinis, C., 2015. First evidence of Leishmania infection in European brown hare (Lepus europaeus) in Greece: GIS analysis and phylogenetic position within the Leishmania spp. Parasitol. Res. https://doi.org/10.1007/s00436-015-4749-8

Yang, B.-B., Guo, X.-G., Hu, X.-S., Zhang, J.-G., Liao, L., Chen, D.-L., Chen, J.-P., 2010. Species discrimination and phylogenetic inference of 17 Chinese Leishmania isolates based on internal transcribed spacer 1 (ITS1) sequences. Parasitol. Res. 107, 1049–1065. https://doi.org/10.1007/s00436-010-1969-9

# CHAPTER V

# THE ROLE OF EUROPEAN BROWN HARE IN THE EPIDEMIOLOGY OF BACTERIAL ZOONOTIC PATHOGENS; A SEROLOGICAL AND MOLECULAR SURVEY IN GREECE

## Abstract

The aim of the present study was to investigate the occurrence of *Bartonella* spp, *Brucella* spp, *Coxiella burnetii*, and *Francisella tularensis* in European Brown hares (*Lepus europaeus*) hunter harvested during two-year hunting periods in northern and central Greece. Serum samples were examined for the presence of IgG antibodies using IFAT and/or ELISA. PCR was used to detect *Bartonella* spp DNA in blood samples and *Brucella* spp, *C. burnetii*, and *F. tularensis DNA* in liver samples. Antibodies against *Bartonella* spp were detected in 12 hares (12/105) while none of the hares examined was seropositive for *Brucella* spp*, C. burnetii*, and *F. tularensis*. The presence of *Bartonella*, *Brucella* spp*, C. burnetii*, and *F. tularensis* DNA was not detected in the samples examined. It seems that the role of European Brown hare in the epidemiology of *Brucella* spp*, C. burnetii*, and *F. tularensis* is of minor importance in Greece while this species is exposed to *Bartonella* spp which gives the impetus for further investigation of its role as another host of this bacterium.

## Introduction

*Bartonella* spp. are facultative intracellular Gram-negative alphaproteobacterial (order *Rhizobiales*, family *Bartonellaceae*) causing bartonellosis worldwide. To date, about 30 *Bartonella* species are known (Birtles, 2005; Silaghi et al., 2016) and distributed among a broad range of mammalian hosts, extending from humans to carnivores, ungulates, rodents, insectivores, bats and lagomorphs (Vayssier-Taussat et al., 2009). Haematophagous arthropod vectors such as fleas, lice, sandflies and ticks have been implicated in the transmission of bartonellae among different mammalian hosts (Bai et al., 2016; Gutiérrez et al., 2015; Silaghi et al., 2016).

Regarding lagomorphs, *Bartonella alsatica* was first isolated from the blood of wild rabbits from the Alsace region in France (Heller et al., 1999). Since then, *B.alsatica* has been also detected in wild rabbits (*Oryctolagus cuniculus*) in Andalusia, Spain (Márquez, 2010) while also two human cases of endocarditis and one human case of lymphadenitis caused by *B.alsatica* have been reported (Angelakis et al., 2008; Jeanclaude et al., 2009; Raoult et al., 2006). *B. alsatica* DNA has been successfully detected in fleas (*Spilopsyllus cuniculi*) from wild rabbits in France and a European wildcat (*Felis silvestris silvestris*) in Andalusia, Spain (MáRquez et al., 2009). This finding suggests that although *S. cuniculi* is a rare infestation in cats, they may get infected through close contact with rabbits and serve as a potential source of *B.alsatica* for humans. On the other hand, a study carried out in California did not detect bartonellae in blood specimens from riparian brush rabbits (*Sylvilagus bachmani riparius*) (Schmitz et al., 2014). Recently, the occurrence of *Bartonella* spp infection in European Brown hare was investigated in central Italy and based on the negative results obtained, the authors suggested that hares may not be very sensitive to these pathogens (Rocchigiani et al., 2018).

*B. abortus*, *B. melitensis*, and *B. suis* are highly pathogenic for domestic and wild animals as well as for humans. Hares have been shown to be infected by *B. suis* biovars 1 and 2 while along with wild boars, they are an important reservoir of *B. suis* biovar 2 (Godfroid et al., 2005; M. Gyuranecz et al., 2011). Although biovar 1 is highly pathogenic and cause severe disease in humans, biovar 2 has only exceptionally been described as the causative agent of human brucellosis (Godfroid et al., 2005; Teyssou et al., 1989). However, the importance of *B. suis* biovar 2 stems for the fact that hares

can maintain the bacterium and infect domestic animals (grazing pigs and cows) even in the absence of a wild boar population (Godfroid et al., 2005).

The wide range of *C. burnetii* host species comprising arthropods, birds and mammals, suggests that complex reservoir systems may exist. Though, domestic ruminants are the main source of *C. burnetii* infection for humans (Meredith et al., 2015). Molecular and serological evidence of *C. burnetii* in hares has been previously reported and attributed to their feeding habits (Astobiza et al., 2011; Ejercito et al., 1993; Marrie et al., 1993).

*F. tularensis* affects more than 300 species of mammals including humans, birds, amphibians or invertebrates, and especially lagomorphs and rodents (Moinet et al., 2016). In the terrestrial lifecycle, which is predominant in most European countries, the lagomorphs, terrestrial rodents and ticks are the main source of human infections (Maurin and Gyuranecz, 2016). European Brown hare is highly susceptible and even extremely low infectious doses result in marked bacteremia thus being a serious source of infection for blood sucking ectoparasites. Moreover, their carcasses and excrements may contaminate the environment while those developing a chronic form of the disease, become a permanent source of *F. tularensis* for other animals in the natural nidus as well as for humans (Treml et al., 2007).

This study aims to investigate the role of European Brown hare in the epidemiology of important bacterial zoonotic pathogens that is, *Bartonella* spp, *Brucella* spp, *C. burnetii* and *F. tularensis* in northern and central Greece.

## Materials and Methods

### Hare samples

Sera (n=105), whole blood samples in EDTA (n=49) and liver samples (n=52) were collected from free-ranged hares that were shot in northern and central Greece (Table 1). Blood samples were collected from the heart or the thoracic cavity of the shot hares. Blood samples without anti-coagulant were centrifuged, and sera was stored at -20°C pending examination for antibodies against *Bartonella* spp, *Brucella* spp*, C. burnetii*, and *F. tularensis*. Blood samples in EDTA and liver samples were stored at -20˚C pending DNA extraction. Sera, blood and liver samples were submitted still frozen to the

Laboratory of Microbiology and Parasitology, University of Thessaly, Greece. Data on gender were recorded, when possible, for the subjects included in the study. The location data of hares were marked with handheld Global Positioning System (GPS) Garmin units in the field.

Table 1. The number of serum, blood and liver hare samples collected in each region of the study area

| Region | Hare serum samples | Hare blood samples | Hare liver samples |
|---|---|---|---|
| Thessaly | 31 | 24 | 27 |
| Central Macedonia, northern Greece | 38 | 15 | 15 |
| Eastern Macedonia and Thrace, northern Greece | 36 | 10 | 10 |
| Total | 105 | 49 | 52 |

## Serological investigation

Serum samples from hares (n=105) were assayed for IgG antibodies against *B.henselae* (Fuller Laboratories Fullerton, California, USA), *B. abortus and F. tularensis* (Fuller Laboratories Fullerton, California, USA) and *C. burnetii* (Vircell, Granada, Spain) using commercial agent specific slides following the manufacturer's instructions. A fluorescein isothiocyanate conjugated sheep anti rabbit IgG (BioFX Laboratories, Owings Mills, USA) was used. Dilutions of the samples were prepared in phosphate buffered saline (PBS). The starting dilution was 1:16, to detect antibodies against *B.henselae*, *Brucella* spp, *C. burnetii* and *F. tularensis*, until the end-point titer. For *Bartonella* spp the endpoint titers defined as the last dilution at which brightly stained organisms could be detected by fluorescence. A cut-off titer for seroreactivity was arbitrarily defined as 1:64. The intensity of bacillus-specific fluorescence was scored subjectively from 1 to 4. Samples with a fluorescence score of ≥2 at a dilution of 1:64

were considered positive. The same two readers performed a double-blind reading of each slide. Titers ≥ 1:32 for *C. burnetii*, *B. abortus* and *F. tularensis* were considered positive. Negative and positive control samples were included on each slide.

The serum samples were also tested using commercial indirect ELISA kits for antibodies against *C. burnetii* (ID Screen® Q Fever Indirect Multi-species, Grabels, France) which included wells coated with a *C. burnetii* phase I and II strain and *Brucella* spp (ID Screen® Brucellosis Serum Indirect Multi-species, Grabels, France) which enables the detection of antibodies against *B. abortus*, *B. melitensis* and *B. suis*. In both ELISA kits, an Anti-multi-species-IgG-HRP conjugate was included and used for the examination of the hare serum samples. All procedures were performed following manufacturer's recommendations.

## Molecular investigation

Total genomic DNA extraction was performed using a commercially available DNA extraction kit (Thermo Scientific GeneJET Genomic DNA Purification Kit, Thermo Fisher Scientific, USA) according to the manufacturer's protocol in 49 blood and 52 liver hare samples. The purified DNA was stored at -20˚C.

*Bartonella* 16S-23S ribosomal RNA intergenic spacer (ITS) PCR amplification was performed as described previously (Vissotto De Paiva Diniz et al., 2007) using the primers 325s 5'-CTT CAG ATG ATG ATC CCA AGC CTT YTG GCG -3' and 1100as 5'-GAA CCG ACG ACC CCC TGC TTG CAA AGC A-3') in DNA extracts from 49 blood samples. Blood samples were further tested for the presence of *Bartonella* DNA using a nested PCR approach (Rampersad et al., 2005) with primers P-bhenfa (5'-TCTTCGTTTCTCTTTCTTCA) and P-benr1 (5'-CAAGCGCGCGCTCTAACC) for the first amplification and nested primers Nbhenf1a (5'-GATGATCCCAAGCCTTCTGGC) and Nbhenr (5'-AACCAACTGAGCTACAAGCC) for the second round of amplification. One microlitre of blood-extracted DNA template was used in the primary PCR reaction and 1 µl of the primary reaction was used in the nested reaction.

The liver hare samples were examined by previously described conventional PCR assays for the detection of *Brucella* spp, *C. burnetii* and *F. tularensis* DNA. *Brucella* 16S rRNA PCR amplification was carried out using the primer pair F4 (5'-TCG AGC GCC CGC AAG GGG-3')-R2 (5'-AAC CAT AGT GTC TCC ACT AA-3') which amplifies a 905-bp fragment,

as described previously (Romero et al., 1995). The Trans-PCR assay was used for the detection of *C.burnetii* DNA with primers Trans1 (5'-TAT GTA TCC ACC GTA GCC AGT C-3') and Trans2 (5'-CCC AAC AAC ACC TCC TTATTC-3') targeting a transposon-like repetitive region of the *C. burnetii* genome (Berri et al., 2000). The expected PCR product size was 687 bp. The 17-kDa gene PCR amplification with primers TUL4-435 and TUL4-863 that yield a 0.4-kb fragment, was performed for the detection of *F. tularensis* DNA (Johansson et al., 2000). Positive and negative controls were included in each PCR run. The PCR products were visualized under UV-light, after 2% agarose gel electrophoresis and staining with ethidium bromide (0.5 μg/ml).

Amplicons from PCR positive hares were sequenced. The positive PCR products were purified using the PureLink PCR purification kit (Invitrogen, Carlsbad, CA, USA) and were bidirectionally sequenced using the fluorescent BigDye Terminator Cycle sequencing kit v3.1 (Applied Biosystems, Foster City, USA), followed by fragment separation with a 3,730xl DNA analyzer (Applied Biosystems, Foster City, USA).

## Ethics

The hare serum, blood and liver samples included in the study, represent material collected opportunistically (no active capture, killing and sampling of wild animals specifically for this study was performed) from animals hunter-harvested by members of the Greek Hunting Federation of Macedonia and Thrace and the Greek Hunting Federation of Thessaly and Sporades, during the hunting seasons, according to the prerequisites of the Greek Legislation and submitted to our laboratory for Passive Wildlife Disease surveillance. Thus, special approval was not necessary and steps to ameliorate suffering were not applicable in this study. Research on animals as defined in the EU Ethics for Researchers document (European Commission, 2007, Ethics for Researchers - Facilitating Research Excellence in FP7, Luxembourg: Office for Official Publications of the European Communities, ISBN 978-92-79-05474-7) is not a part of the study.

## Results

### Serological investigation

Antibodies against *Bartonella* spp were detected in 12 hare serum samples (12/105, 11.4% 95% CI: 6.6-18.9); 11 samples from northern Greece (11/74, 14.8% 95% CI: 8.5-24.6) and one sample (1/31, 3.2% 95% CI: 0.5-16.1) from central Greece (Table 2). Titers of antibodies against *Bartonella* spp ranged from 1:64 to 1:128; serum titers of 1:64 in 11 hares and 1:128 in one hare. Although 19 additional hare serum samples (10 from northern Greece and nine from central Greece) were found seroreactive in 1:32 dilution, they were designated as negative based on the cut-off value used and they were not included in the seroprevalence estimation. None of the hare serum samples was found positive for antibodies against *C. burnetii* and *Brucella* spp by IFAT and ELISA and *F. tularensis* by IFAT.

Records on the gender were available for 39 out of the 105 subjects (17 males and 22 females). Out of the 12 *Bartonella* seropositive hares, gender was recorded for five individuals; two males and three females. The gender was unknown for the remaining seven seropositive individuals.

Table 2. The prevalence of antibodies against *Bartonella spp, Brucella spp, C.burnetii,* and *F.tularensis* in European brown hares from central and northern Greece

| Region | Hare serum samples | *Bartonella spp* | *Brucella* spp | *C. burnetii* | *F. tularensis* |
|---|---|---|---|---|---|
| | | Positive | Positive | Positive | Positive |
| Thessaly | 31 | 1 | 0 | 0 | 0 |
| Central Macedonia, northern Greece | 38 | 4 | 0 | 0 | 0 |
| Eastern Macedonia and Thrace, northern Greece | 36 | 7 | 0 | 0 | 0 |
| Total | 105 | 12 | 0 | 0 | 0 |

## Molecular investigation

None of the blood samples examined was positive for *Bartonella* DNA in the 16S-23S ribosomal RNA intergenic spacer (ITS) PCR amplification nor in the nested approach. Similarly, none of the examined hare liver samples was found positive for *Brucella* spp, *C. burnetii*, and *F. tularensis* DNA.

## Discussion

This study reports the occurrence of *Bartonella* spp exposure of European brown hares in northern and central Greece with an overall seroprevalence of 11.4% whereas none of the hares examined was seropositive for *Brucella* spp, *C. burnetii* and *F. tularensis*. *Bartonella* spp, *Brucella* spp, *C. burnetii* and *F. tularensis* DNA was not detected in any of the samples examined.

Serology can be used to infer previous exposure of a host to *Bartonella* spp. However, it is highly insensitive for species identification due to cross reactivity to antigens common to most *Bartonella* species (Chomel et al., 2004; MacDonald et al., 2004). In this study, the occurrence of hare exposure to *Bartonella* spp is documented through the detection of IgG antibodies in 11.4% of the hare serum samples examined using IFAT slides coated with cells infected with *B.henselae.* The seropositive results obtained are possibly justified by the cross reactivity to common antigens among *Bartonella* species. A low level of cross-reaction with *C. burnetii* has been also reported in *Bartonella* infections (La Scola and Raoult, 1996). However, none of the hares examined was seroreactive for *C.burnetii* in this study.

Because of cross-reactivity, bacterial isolation or PCR assays are necessary to identify the infecting *Bartonella* spp (La Scola and Raoult, 1996; MacDonald et al., 2004). Furthermore, enrichment culture of clinical specimens in an optimized, insect cell culture growth medium, prior to PCR testing, substantially increase the sensitivity of detecting *Bartonella* infection (Breitschwerdt, 2017). The blood samples included in this study were collected from the heart or the thoracic cavity of the shot hares by hunters and foresters and thus, the collection process was not strictly controlled, and it is not considered aseptic. This is the reason why culture was not attempted in our study; fastidious growth requirements of this genus of bacteria are well known while slow growth and long culture time for *Bartonella* spp together with the faster growing

contaminating bacteria and fungi that can overrun a culture plate, can make the detection of *Bartonella* difficult or even impossible (Rampersad et al., 2005). In our study, *Bartonella* DNA failed to be amplified using PCR in hare blood samples. The decisive factors for this result may be the small amount of detectable *Bartonella* DNA in the hare blood samples, the small number of samples examined and the lack of hare competence as a reservoir host for *Bartonella* spp. Similar results were also obtained previously in central Italy where none of the examined European brown hares was found positive for *Bartonella* DNA (Rocchigiani et al., 2018). Further studies, using culture or enrichment culture of clinical specimens prior to PCR testing are needed to investigate the occurrence of *Bartonella* DNA in hare samples. Moreover, the study design should definitely include strict protocols for sample collection so as to ensure the aseptic conditions during sampling and make possible the bacterial culture.

Albeit not being able to demonstrate the occurrence of *Bartonella* DNA in hare blood samples and to define the responsible species, our findings are suggestive of hare exposure to *Bartonella* spp. Hare is often infested with ectoparasites including fleas, ticks and lice (Dik and Uslu, 2018) but data on the arthropod species infesting hares in Greece are lacking. It has been suggested that there is not a specific "hare flea" for European hares, but rabbit, rat or hedgehog fleas may infest this species (Mullineaux et al., 2016). European brown hare is a secondary host of *S. cuniculi* which is widely distributed following its primary host, the rabbit, and it has been implicated in the transmission cycle of *B.alsatica* in wild rabbits, cats and humans. Various mammals including red fox, cat, dog, rodents, mustelids, voles, marten, badger and wild boars are accidental hosts of *S. cuniculi. Pulex irritans* and *Nosopsyllus fasciatus* have been also detected in European brown hare (Dik and Uslu, 2018) while also they attack a wide variety of mammals including rodents and humans and they are competent vectors of *Bartonella* spp (Bitam et al., 2010). Among the tick genera reported in hares, *Ixodes*, *Haemaphysalis* and *Dermacentor* (Dantas-Torres et al., 2011; Dik and Uslu, 2018; Psaroulaki et al., 2014) were found infected by several *Bartonella* spp (Breitschwerdt, 2017; Sréter-Lancz et al., 2006) in previous studies but a causal link between *Bartonella* spp., clinical signs, and tick bite has not been established yet (Vayssier-Taussat et al., 2016). Although, further studies are needed to infer the role of hare in the transmission cycle of bartonellae, the results obtained so far are not

suggestive of its role as an alternative reservoir. Though, hare might play a role in the eco-epidemiology of *Bartonella* spp as a maintainer or carrier of infected vectors. Although the detected antibody titers were quite low, the presence of antibodies against *Bartonella* spp indicate exposure to the pathogen possibly following ectoparasite infestation. Several factors may affect the prevalence of antibodies against *Bartonella* spp and the antibody response in a specific host, including the level of infestation with the relevant vector, the population density, the hare's possible resistance to infection, the seasonal activity patterns of vectors and even the specific *Bartonella* species present as it has been suggested for rodents (Tomassone et al., 2018). However, all these factors should be further investigated so as to understand the hare-*Bartonella* interaction. The importance of hare in the epidemiology of vector borne diseases such as bartonellosis, stems for the fact that hares, while keeping their small home range, share the same ectoparasites and habitat with other domestic and wild animals, some of which may be important hosts of *Bartonella* spp or even reservoirs of bartonellae such as cats and rodents. The latter are the preferential reservoir hosts of several *Bartonella* species in nature among which, *B. elizabethae*, associated with the black rat and Oriental rat fleas (*Xenopsylla cheopis*), and *B. grahamii*, associated with wild mice and voles and transmitted by rodent fleas, are recognized as zoonotic pathogens (Chomel and Kasten, 2010). Moreover, the pathogenic *B. quintana* and *B. koehlerae* have been detected in rodent fleas (Marié et al., 2006). This is further complicated considering that fleas only show host preference but no clear host specificity (Silaghi et al., 2016).

Reported seroprevalences for *Brucella* spp in hares range from 0% to 17% (Winkelmayer et al., 2005). The absence of antibodies against *Brucella* spp found in this study, is consistent with previous studies conducted in Czech Republic (Hubálek et al., 1993) and Germany (Frandölich et al., 2003) suggesting that *Le.europaeus* plays no significant role as wildlife reservoir for *B. suis* in Greece. We could also hypothesize that the hare population examined had no previous or current contact with infected wild boars or infected domestic pigs. Besides, to our knowledge, there are no studies on the seroprevalence of *B. suis* in wild boars in Greece while domestic pigs are mainly kept indoors and a serosurvey conducted previously in swine herds in northern and southern Greece showed a 3% seroprevalence (Burriel et al., 2003).

Our findings on the absence of antibodies against *C. burnetii* are in agreement with studies previously conducted in Czech Republic (Hubálek et al., 1993) and Germany (Dedek et al., 1990). However, a seroprevalence of 49% in hares was reported previously in Nova Scotia (Marrie et al., 1993). Serological studies in Greece reported seropositivity for *C. burnetti* in 35% of the dairy cattle herds examined in central and northern Greece (Dovolou et al., 2011), in 6.5% of goats and 10.4% of sheep in Northern Greece (Pape et al., 2009) and in 14.5% of sheep and 15% of goats in Central Greece (Valiakos et al., 2017). A possible explanation for the negative results of our study is that the hare population examined in our study did not get infected when fed on pastures that semi-extensive livestock animals have access in summer and could possibly contaminate them with *C. burnetii* positive faeces (Ejercito et al., 1993).

Serological tests are useful diagnostic tools for epidemiological surveys of tularemia in European brown hares as they are moderately sensitive to *F. tularensis* infection, they seroconvert, and they can potentially carry viable bacteria over a longer time span and thus serve as a reservoir species (Mikl?s Gyuranecz et al., 2011). Previous studies in other European countries showed that the seroprevalence of *F. tularensis* in hares range from 4.5% (Hoflechner-Poltl, 2000) to 6.5% (Treml et al., 2007). To our knowledge, there are no studies on *F. tularensis* in Greece. The negative serological findings on the hare population from this study are consistent with the existing knowledge that Greece is among the European countries that are deemed to be free of tularaemia (Maurin and Gyuranecz, 2016).

## Conclusions

Our fidings support the minor importance of the role of European Brown hare in the epidemiology of *Brucella* spp*, C. burnetii*, and *F. tularensis* in Greece and are suggestive of European brown hare exposure to *Bartonella* spp. The studies conducted so far, support the lack of hare competence as a reservoir host for *Bartonella* spp. It is possible that hare acts as a maintainer or carrier of infected vectors, but this hypothesis remains to be confirmed by other studies. The need for culture or enrichment culture of clinical specimens prior to PCR testing and aseptic sampling in order to draw solid conclusions on the presence or absence of *Bartonella* DNA in clinical samples are herein highlighted and should be considered in future studies.

## References


Angelakis, E., Lepidi, H., Canel, A., Rispal, P., Perraudeau, F., Barre, I., Rolain, J.-M., Raoult, D., 2008. Human Case of *Bartonella alsatica* Lymphadenitis. Emerg. Infect. Dis. 14, 1951–1953. https://doi.org/10.3201/eid1412.080757

Astobiza, I., Barral, M., Ruiz-Fons, F., Barandika, J.F., Gerrikagoitia, X., Hurtado, A., García-Pérez, A.L., 2011. Molecular investigation of the occurrence of Coxiella burnetii in wildlife and ticks in an endemic area. Vet. Microbiol. 147, 190–194. https://doi.org/10.1016/j.vetmic.2010.05.046

Bai, Y., Gilbert, A., Fox, K., Osikowicz, L., Kosoy, M., 2016. *BARTONELLA ROCHALIMAE* AND *B. VINSONII* SUBSP. *BERKHOFFII* IN WILD CARNIVORES FROM COLORADO, USA. J. Wildl. Dis. 52, 844–849. https://doi.org/10.7589/2016-01-015

Berri, M., Laroucau, K., Rodolakis, A., 2000. The detection of Coxiella burnetii from ovine genital swabs, milk and fecal samples by the use of a single touchdown polymerase chain reaction. Vet. Microbiol. 72, 285–293.

Birtles, R.J., 2005. Bartonellae as Elegant Hemotropic Parasites. Ann. N. Y. Acad. Sci. 1063, 270–279. https://doi.org/10.1196/annals.1355.044

Bitam, I., Dittmar, K., Parola, P., Whiting, M.F., Raoult, D., 2010. Fleas and flea-borne diseases. Int. J. Infect. Dis. 14, e667–e676. https://doi.org/10.1016/j.ijid.2009.11.011

Breitschwerdt, E.B., 2017. Bartonellosis, One Health and all creatures great and small. Vet. Dermatol. 28, 96-e21. https://doi.org/10.1111/vde.12413

Burriel, A.R., Varoudis, L., Alexopoulos, C., Kritas, S., Kyriakis, S.C., 2003. Serological evidence of Brucella species and Leptospira interrogans serovars in Greek swine herds. J Swine Health Prod 11, 186–189.

Chomel, B.B., Boulouis, H.J., Breitschwerdt, E.B., 2004. Cat scratch disease and other zoonotic Bartonella infections. J. Am. Vet. Med. Assoc. 224, 1270–1279.

Chomel, B.B., Kasten, R.W., 2010. Bartonellosis, an increasingly recognized zoonosis: Bartonellosis, an increasingly recognized zoonosis. J. Appl. Microbiol. 109, 743–750. https://doi.org/10.1111/j.1365-2672.2010.04679.x

Dantas-Torres, F., Testini, G., DiGeronimo, P.M., Lorusso, V., Mallia, E., Otranto, D., 2011. Ticks infesting the endangered Italian hare (Lepus corsicanus) and their

habitat in an ecological park in southern Italy. Exp. Appl. Acarol. 53, 95–102. https://doi.org/10.1007/s10493-010-9387-3

Dedek, J., Nattermann, H., Loepelmann, H., Rinka, E., Knopke, C., 1990. RESULTS OBTAINED FROM SEROLOGICAL INVESTIGATIONS OF HARE (LEPUS-EUROPAEUS-PALLAN, 1778) FOR SELECTED INFECTIONS. Monatshefte fur veterinarmedizin 45, 833–836.

Dik, B., Uslu, U., 2018. Ectoparasites of hares (Lepus europaeus Pallas) in Konya Province, Turkey. Turk. J. Vet. Anim. Sci. 42, 65–72. https://doi.org/10.3906/vet-1706-19

Dovolou, E., Tsiligianni, T., Vouzaras, D., Amiridis, G.S., 2011. Prevalence of Coxiella burnetii antibodies in bulk milk and blood serum and associations with reproductive indices in cow dairy herds of Central and Northern Greece. J Hellenic Vet Med Soc 62, 314–319.

Ejercito, C.L.A., Cai, L., Htwe, K.K., Taki, M., Inoshima, Y., Kondo, T., Kano, C., Abe, S., Shirota, K., Sugimoto, T., Yamaguchi, T., Fukushi, H., Minamoto, N., Kinjo, T., Isogai, E., Hirai, K., 1993. Serological Evidence of Coxiella burnetii Infection in Wild Animals in Japan. J. Wildl. Dis. 29, 481–484. https://doi.org/10.7589/0090-3558-29.3.481

Frandölich, K., Wisser, J., Schmüser, H., Fehlberg, U., Neubauer, H., Grunow, R., Nikolaou, K., Priemer, J., Thiede, S., Streich, W.J., Speck, S., 2003. EPIZOOTIOLOGIC AND ECOLOGIC INVESTIGATIONS OF EUROPEAN BROWN HARES (LEPUS EUROPAEUS) IN SELECTED POPULATIONS FROM SCHLESWIG-HOLSTEIN, GERMANY. J. Wildl. Dis. 39, 751–761. https://doi.org/10.7589/0090-3558-39.4.751

Godfroid, J., Cloeckaert, A., Liautard, J.-P., Kohler, S., Fretin, D., Walravens, K., Garin-Bastuji, B., Letesson, J.-J., 2005. From the discovery of the Malta fever?s agent to the discovery of a marine mammal reservoir, brucellosis has continuously been a re-emerging zoonosis. Vet. Res. 36, 313–326. https://doi.org/10.1051/vetres:2005003

Gutiérrez, R., Krasnov, B., Morick, D., Gottlieb, Y., Khokhlova, I.S., Harrus, S., 2015. *Bartonella* Infection in Rodents and Their Flea Ectoparasites: An Overview. Vector-Borne Zoonotic Dis. 15, 27–39. https://doi.org/10.1089/vbz.2014.1606

Gyuranecz, M., Erdélyi, K., Makrai, L., Fodor, L., Szépe, B., Mészáros, Á.R., Dán, A., Dencső, L., Fassang, E., Szeredi, L., 2011. Brucellosis of the European Brown Hare (Lepus europaeus). J. Comp. Pathol. 145, 1–5. https://doi.org/10.1016/j.jcpa.2010.11.013

Gyuranecz, Mikl?s, Rig?, K., D?n, ?d?m, F?ldv?ri, G., Makrai, L., D?nes, B., Fodor, L., Majoros, G., Tirj?k, L., Erd?lyi, K., 2011. Investigation of the Ecology of *Francisella tularensis* During an Inter-Epizootic Period. Vector-Borne Zoonotic Dis. 11, 1031–1035. https://doi.org/10.1089/vbz.2010.0091

Heller, R., Kubina, M., Mariet, P., Riegel, P., Delacour, G., Dehio, C., Lamarque, F., Kasten, R., Boulouis, H.-J., Monteil, H., Chomel, B., Piemont, Y., 1999. Bartonella alsatica sp. nov., a new Bartonella species isolated from the blood of wild rabbits. Int. J. Syst. Bacteriol. 49, 283–288. https://doi.org/10.1099/00207713-49-1-283

Hubálek, Z., Juricová, Z., Svobodová, S., Halouzka, J., 1993. A serologic survey for some bacterial and viral zoonoses in game animals in the Czech Republic. J. Wildl. Dis. 29, 604–607. https://doi.org/10.7589/0090-3558-29.4.604

Jeanclaude, D., Godmer, P., Leveiller, D., Pouedras, P., Fournier, P.-E., Raoult, D., Rolain, J.-M., 2009. Bartonella alsatica endocarditis in a French patient in close contact with rabbits. Clin. Microbiol. Infect. 15, 110–111. https://doi.org/10.1111/j.1469-0691.2008.02187.x

Johansson, A., Berglund, L., Eriksson, U., Göransson, I., Wollin, R., Forsman, M., Tärnvik, A., Sjöstedt, A., 2000. Comparative analysis of PCR versus culture for diagnosis of ulceroglandular tularemia. J. Clin. Microbiol. 38, 22–26.

La Scola, B., Raoult, D., 1996. Serological cross-reactions between Bartonella quintana, Bartonella henselae, and Coxiella burnetii. J. Clin. Microbiol. 34, 2270–2274.

MacDonald, K.A., Chomel, B.B., Kittleson, M.D., Kasten, R.W., Thomas, W.P., Pesavento, P., 2004. A prospective study of canine infective endocarditis in northern California (1999-2001): emergence of Bartonella as a prevalent etiologic agent. J. Vet. Intern. Med. 18, 56–64.

Marié, J.-L., Fournier, P.-E., Rolain, J.-M., Briolant, S., Davoust, B., Raoult, D., 2006. Molecular detection of Bartonella quintana, B. Elizabethae, B. Koehlerae, B.

Doshiae, B. Taylorii, and Rickettsia felis in rodent fleas collected in Kabul, Afghanistan. Am. J. Trop. Med. Hyg. 74, 436–439.

Márquez, F.J., 2010. Molecular detection of Bartonella alsatica in European wild rabbits (Oryctolagus cuniculus) in Andalusia (Spain). Vector Borne Zoonotic Dis. Larchmt. N 10, 731–734. https://doi.org/10.1089/vbz.2009.0135

MáRquez, F.J., MilláN, J., RodríGuez-LiéBana, J.J., GarcíA-Egea, I., Muniain, M.A., 2009. Detection and identification of *Bartonella* sp. in fleas from carnivorous mammals in Andalusia, Spain. Med. Vet. Entomol. 23, 393–398. https://doi.org/10.1111/j.1365-2915.2009.00830.x

Marrie, T.J., Embil, J., Yates, L., 1993. Seroepidemiology of Coxiella burnetii among wildlife in Nova Scotia. Am. J. Trop. Med. Hyg. 49, 613–615.

Maurin, M., Gyuranecz, M., 2016. Tularaemia: clinical aspects in Europe. Lancet Infect. Dis. 16, 113–124. https://doi.org/10.1016/S1473-3099(15)00355-2

Meredith, A.L., Cleaveland, S.C., Denwood, M.J., Brown, J.K., Shaw, D.J., 2015. *Coxiella burnetii* (Q-Fever) Seroprevalence in Prey and Predators in the United Kingdom: Evaluation of Infection in Wild Rodents, Foxes and Domestic Cats Using a Modified ELISA. Transbound. Emerg. Dis. 62, 639–649. https://doi.org/10.1111/tbed.12211

Moinet, M., Decors, A., Mendy, C., Faure, E., Durand, B., Madani, N., 2016. Spatio-temporal dynamics of tularemia in French wildlife: 2002–2013. Prev. Vet. Med. 130, 33–40. https://doi.org/10.1016/j.prevetmed.2016.05.015

Mullineaux, E., Keeble, E.J., British Small Animal Veterinary Association, 2016. BSAVA manual of wildlife casualties.

Pape, M., Bouzalas, E.G., Koptopoulos, G.S., Mandraveli, K., Arvanitidou-Vagiona, M., Nikolaidis, P., Alexiou-Daniel, S., 2009. The serological prevalence of Coxiella burnetii antibodies in sheep and goats in northern Greece. Clin. Microbiol. Infect. 15, 146–147. https://doi.org/10.1111/j.1469-0691.2008.02159.x

Psaroulaki, A., Chochlakis, D., Angelakis, E., Ioannou, I., Tselentis, Y., 2014. Coxiella burnetii in wildlife and ticks in an endemic area. Trans. R. Soc. Trop. Med. Hyg. 108, 625–631. https://doi.org/10.1093/trstmh/tru134

Rampersad, J.N., Watkins, J.D., Samlal, M.S., Deonanan, R., Ramsubeik, S., Ammons, D.R., 2005. A nested-PCR with an Internal Amplification Control for the

detection and differentiation of Bartonella henselae and B. clarridgeiae: An examination of cats in Trinidad. BMC Infect. Dis. 5. https://doi.org/10.1186/1471-2334-5-63

Raoult, D., Roblot, F., Rolain, J.-M., Besnier, J.-M., Loulergue, J., Bastides, F., Choutet, P., 2006. First Isolation of Bartonella alsatica from a Valve of a Patient with Endocarditis. J. Clin. Microbiol. 44, 278–279. https://doi.org/10.1128/JCM.44.1.278-279.2006

Rocchigiani, G., Ebani, V.V., Nardoni, S., Bertelloni, F., Bascherini, A., Leoni, A., Mancianti, F., Poli, A., 2018. Molecular survey on the occurrence of arthropod-borne pathogens in wild brown hares ( Lepus europaeus ) from Central Italy. Infect. Genet. Evol. 59, 142–147. https://doi.org/10.1016/j.meegid.2018.02.005

Romero, C., Gamazo, C., Pardo, M., López-Goñi, I., 1995. Specific detection of Brucella DNA by PCR. J. Clin. Microbiol. 33, 615–617.

Schmitz, K.M., Foley, J.E., Kasten, R.W., Chomel, B.B., Larsen, R.S., 2014. Prevalence of Vector-borne Bacterial Pathogens in Riparian Brush Rabbits ( *Sylvilagus bachmani riparius* ) and their Ticks. J. Wildl. Dis. 50, 369–373. https://doi.org/10.7589/2012-11-292

Silaghi, C., Pfeffer, M., Kiefer, D., Kiefer, M., Obiegala, A., 2016. Bartonella, Rodents, Fleas and Ticks: a Molecular Field Study on Host-Vector-Pathogen Associations in Saxony, Eastern Germany. Microb. Ecol. 72, 965–974. https://doi.org/10.1007/s00248-016-0787-8

Sréter-Lancz, Z., Tornyai, K., Széll, Z., Sréter, T., Márialigeti, K., 2006. Bartonella infections in fleas (Siphonaptera: Pulicidae) and lack of bartonellae in ticks (Acari: Ixodidae) from Hungary. Folia Parasitol. (Praha) 53, 313–316.

Teyssou, R., Morvan, J., Leleu, J.P., Roumegou, P., Goullin, B., Carteron, B., 1989. A propos d'un cas de brucellose humaine a Brucella suis biovar 2. Mdecine Mal. Infect. 19, 160–161. https://doi.org/10.1016/S0399-077X(89)80221-5

Tomassone, L., Berriatua, E., De Sousa, R., Duscher, G.G., Mihalca, A.D., Silaghi, C., Sprong, H., Zintl, A., 2018. Neglected vector-borne zoonoses in Europe: Into the wild. Vet. Parasitol. 251, 17–26. https://doi.org/10.1016/j.vetpar.2017.12.018

Treml, F., Pikula, J., Bandouchova, H., Horakova, J., 2007. European brown hare as a potential source of zoonotic agents. Vet. Med. (Praha) 52, 451–456.

Valiakos, G., Giannakopoulos, A., Spanos, S.A., Korbou, F., Chatzopoulos, D.C., Mavrogianni, V.S., Spyrou, V., Fthenakis, G.C., Billinis, C., 2017. Use of geographical information system and ecological niche model to analyse potential exposure of small ruminants to Coxiella burnetii infection in central Greece. Small Rumin. Res. 147, 77–82. https://doi.org/10.1016/j.smallrumres.2016.12.042

Vayssier-Taussat, M., Le Rhun, D., Bonnet, S., Cotté, V., 2009. Insights in Bartonella Host Specificity. Ann. N. Y. Acad. Sci. 1166, 127–132. https://doi.org/10.1111/j.1749-6632.2009.04531.x

Vayssier-Taussat, M., Moutailler, S., Féménia, F., Raymond, P., Croce, O., La Scola, B., Fournier, P.-E., Raoult, D., 2016. Identification of Novel Zoonotic Activity of Bartonella spp., France. Emerg. Infect. Dis. 22, 457–462. https://doi.org/10.3201/eid2203.150269

Vissotto De Paiva Diniz, P.P., Maggi, R.G., Schwartz, D.S., Cadenas, M.B., Bradley, J.M., Hegarty, B., Breitschwerdt, E.B., 2007. Canine bartonellosis: serological and molecular prevalence in Brazil and evidence of co-infection with *Bartonella henselae* and *Bartonella vinsonii* subsp. *berkhoffii*. Vet. Res. 38, 697–710. https://doi.org/10.1051/vetres:2007023

Winkelmayer, R., Vodnansky, M., Paulsen, P., Gansterer, A., Treml, F., 2005. Explorative study on the seroprevalence of Brucella-, Francisella- and Leptospira antibodies in the European hare (Lepus europaeus Pallas) of the Austrian - Czech border region. Vet. Med. Austria 92, 131–135.

# GENERAL CONCLUSIONS

# GENERAL CONCLUSIONS

The studies conducted in this PhD thesis led to the following conclusions:

- *Leishmania* infection was evidenced for the first time in European brown hare in Greece. The *Leishmania* DNA prevalence was found to be 23.49% in brown hares from Thessaloniki and Chalkidiki during 2007-2011. The high prevalence of infection found in our study together with the infectiousness of hares to sand flies and the feeding preference of the latter for hares, as documented earlier in Spain, raises concern about the role of hares in the epidemiology of *Leishmania spp* in Greece. The nucleotide sequence homology detected between the hare and the canine *Leishmania* sequences from the same areas was 98.9%, indicating a possible overlapping of wild and domestic transmission cycles. No association between human leishmaniasis cases and hare infection was indicated and the hypothesis made was that leishmaniasis is still in the sylvatic transmission cycle in Chalkidiki whereas it seems that an overlapping of sylvatic and domestic transmission cycles occurs in Thessaloniki. Precipitation was recognized as an important environmental parameter influencing the probability of a hare to be *Leishmania* infected.
- The occurrence of *T.gondii* (5.7%), *N.caninum* (0.95%) and *L.infantum* (12.4%) exposure and the *N.caninum* (3.8%) and *L.infantum* (9.6%) infection in European brown hares from central and northern Greece, were demonstrated for the first time. The low *N.caninum* seroprevalence (0.95%) and DNA prevalence (3.8%) indicated the existing although limited exposure of this species to *N.caninum* in Greece and a low risk for hunting dogs, wild canids and wild boar to be infected through the consumption of infected hares. The most important influential environmental variables associated with hare exposure to *T.gondii* and *L.infantum* were precipitation and habitat types. These parameters affect a) the dynamics of oocysts survival and dispersal for *T.gondii*, b) the activity period of sandflies, their survival, the promotion of adult emergence and the determination of the appropriate oviposition sites for *L.infantum* and c) the population density of important hosts in both cases. The overlapping of sylvatic and domestic transmission cycles of *L.infantum* was suggested for the

region of Thessaly as well, a highly endemic region for human and canine leishmaniasis, where *Leishmania* infection has been also reported in cats and red foxes. This hypothesis was further supported by the exposure of hares to *N. caninum* and *T. gondii* that indirectly indicated that hares share the same living space with their definitive hosts, that is wild carnivores such as gray wolf and dogs *for N. caninum* and cats or free-ranging felids for *T. gondii.* Concerning the hare-red fox contact, it is well documented as hare is a common prey for red foxes.

- *Leishmania* sequences derived from different hosts in Thessaly, that is humans, dogs, cats and hares, belonged in the *L.donovani* complex. *L. infantum* was identified in all the *Leishmania* sequences included in the study suggesting the predominance of this species, the existence of the zoonotic transmission cycle of *L.infantum* and the possible overlapping between domestic and sylvatic transmission cycles in the study area. *L.donovani* was identified in one feline *Leishmania* sequence, providing indication of the possible circulation of a previously unreported *Leishmania* species in Greece which should be further investigated in future studies.
- The role of European Brown hare in the epidemiology of *Brucella* spp*, C. burnetii*, and *F. tularensis* is of minor importance in Greece as suggested by the negative serological and molecular results obtained. Though, the exposure of European brown hares to *Bartonella* spp (11.4%) which was reported for the first time, gives the impetus for further investigation of its role as another host of this bacterium. These findings are not suggestive of its role as an alternative reservoir, but this species may act as a maintainer or carrier of infected vectors. Besides, while keeping their small home range, hares share the same ectoparasites and habitat with other domestic and wild animals, some of which may be important hosts of *Bartonella* spp or even reservoirs of bartonellae such as cats and rodents.

This thesis gives insight into the role of an important game species in Greece, European brown hare, in the epidemiology of parasitic and bacterial pathogens of zoonotic potential. This species has a limited home range, being thus prone to many

infectious diseases of natural nidality while sharing the same living space with other wild and free-ranging animals including canids, felids and large birds of prey, which comprise its natural predators and hunting grounds with domestic dogs. Its importance is further enhanced by cross border and long-distance movements possibly as part of live animal translocations, as the host of vectors and the prey of carnivores and omnivores that travel in great distances and get into contact with domestic animals and humans. Besides, its short life span makes this species an excellent indicator for recent pathogen transmission in the area and its intensive contact with vectors such as ticks, mosquitoes and sandflies has implicated hare in the epidemiology of important vector borne diseases such as Leishmaniasis. The continuous surveillance of hare populations could provide information on the hare population health status and the pathogens circulating in the area posing risk for wildlife, domestic animals and humans.

UNIVERSITY OF THESSALY

SCHOOL OF HEALTH SCIENCES

FACULTY OF VETERINARY MEDICINE

DEPARTMENT OF MICROBIOLOGY AND PARASITOLOGY

# THE ROLE OF EUROPEAN BROWN HARE (*LEPUS EUROPAEUS*) AS A SOURCE OF EMERGING AND RE-EMERGING PATHOGENS

Doctoral Thesis by

Constantina N. Tsokana

## Summary

This thesis aimed to investigate the role of European brown hare (*Lepus europaeus*) as a source of important emerging and re-emerging pathogens. The serological and molecular surveys herein conducted investigated for the first time the occurrence of infection and/or exposure of brown hare to *Toxoplasma gondii, Neospora caninum*, *Leishmania infantum, Bartonella* spp, *Brucella* spp, *Coxiella burnetii*, and *Francisella tularensis* in Greece. Moreover, the Ecological Niche of *Toxoplasma gondii* and *Leishmania infantum* in seropositive hares has been defined. The role of this species in the epidemiology of Leishmaniasis was further investigated through GIS analysis for identification of the environmental parameters related to *Leishmania* infection in hares as well as phylogenetic analysis of the *Leishmania* parasites detected in hares. The phylogenetic position of the *Leishmania* spp detected in humans, dogs, cats and hares in Thessaly, Central Greece and the first indication for the circulation of *L.donovani* in Greece are also part of this thesis.

In the first chapter of this thesis the tools that have been developed for molecular diagnosis of Leishmaniasis, species identification and phylogenetic analysis presenting different sensitivity and discriminatory power were summarized.

The first evidence of *Leishmania* infection in European Brown Hare in Greece was reported in the second chapter of this thesis. The *Leishmania* DNA prevalence in hares was found to be 23.49% in Northern Greece (prefectures of Thessaloniki and Chalkidiki) from 2007-2011. The phylogenetic analysis showed that the hare *Leishmania* sequences belong in the *Leishmania donovani* complex. The homology of the nucleotide sequences with canine *Leishmania* sequences of the same area was 98.9% suggesting a possible overlapping of wild and domestic transmission cycles of *Leishmania spp* in the region. There was no indication that there is an association between the human leishmaniasis cases and the infected hares in the prefectures studied while the hypothesis made was that leishmaniasis is still in the sylvatic transmission cycle in Chalkidiki whereas it seems that an overlapping of sylvatic and domestic transmission cycles occurs in Thessaloniki. Precipitation was recognized as the environmental parameter influencing the presence of *Leishmania* DNA in hares with an increase of one unit in the precipitation seasonality to be expected to increase the odds of hares to be *Leishmania* positive by 6.1%.

The third chapter concerns the serological and molecular survey for parasitic pathogens in European brown hares from Northern and central Greece during a two year hunting period which showed that the examined hares were *N.caninum* (3.8%) and *L.infantum* (9.6%) infected while, 5.7%, 0.95% and 12.4% of the hares reacted positively for the presence of antibodies against *T.gondii, N.caninum* and *L.infantum* respectively. Geographical information system (GIS) analysis together with the Ecological Niche Model (ENM) showed that rainfall indices and land uses are significantly influential for hare exposure to *T.gondii* and *L.infantum*. The high-risk areas for hare exposure to *T.gondii* and *L.infantum* were identified and a risk map for the regions of northern and central Greece was created.

The phylogenetic position of *Leishmania* spp detected in humans, dogs, cats and hares in Thessaly, Central Greece was defined through phylogenetic and analysis of ITS1 for microsatellite repeat numbers and it is described in the fourth chapter of this thesis. The *Leishmania* sequences derived from different hosts belonged in the *L. donovani*

complex. *L. infantum* was identified in all the *Leishmania* sequences included in the study supporting the predominance of *L.infantum* circulation in different hosts in Thessaly, the existence of the zoonotic transmission cycle of *L.infantum* in the study area and the possible overlapping between domestic and sylvatic transmission cycles. Unexpectedly, one *Leishmania* sequence was identified as *L. donovani* in one feline sample indicating the possible *L. donovani* infection in a cat from the study area giving the impetus for further investigation.

The last chapter concerns the occurrence of infection and/or exposure of European Brown hares to *Bartonella* spp, *Brucella* spp, *Coxiella burnetii*, and *Francisella tularensis* which are investigated during two-year hunting periods in northern and central Greece. Antibodies against *Bartonella* spp were detected in 11.4% of the examined hares while none of them was seropositive for *Brucella* spp*, C. burnetii*, and *F. tularensis.* The presence of *Bartonella*, *Brucella* spp*, C. burnetii*, and *F. tularensis* DNA was not detected in the samples examined. It seems that the role of European Brown hare in the epidemiology of *Brucella* spp*, C. burnetii*, and *F. tularensis* is of minor importance in Greece while this species is exposed to *Bartonella* spp which gives the impetus for further investigation of its role as another host of this bacterium.

This thesis gives insight into the role of an important game species in Greece, European brown hare, in the epidemiology of parasitic and bacterial pathogens of zoonotic potential. This species has a limited home range, being thus prone to many infectious diseases of natural nidality while sharing the same living space with other wild and free-ranging animals including canids, felids and large birds of prey, which comprise its natural predators and hunting grounds with domestic dogs. Its importance is further enhanced by cross border and long-distance movements possibly as part of live animal translocations, as the host of vectors and the prey of carnivores and omnivores that travel in great distances and get into contact with domestic animals and humans. Besides, its short life span makes this species an excellent indicator for recent pathogen transmission in the area and its intensive contact with vectors such as ticks, mosquitoes and sandflies has implicated hare in the epidemiology of important vector borne diseases such as Leishmaniasis. The continuous surveillance of hare populations could provide information on the population health status and the pathogens circulating in the area posing risk for wildlife, domestic animals and humans.

ΠΑΝΕΠΙΣΤΗΜΙΟ ΘΕΣΣΑΛΙΑΣ

ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ

ΤΜΗΜΑ ΚΤΗΝΙΑΤΡΙΚΗΣ

ΕΡΓΑΣΤΗΡΙΟ ΜΙΚΡΟΒΙΟΛΟΓΙΑΣ ΚΑΙ ΠΑΡΑΣΙΤΟΛΟΓΙΑΣ

# Ο ΡΟΛΟΣ ΤΟΥ ΚΑΦΕ ΕΥΡΩΠΑΪΚΟΥ ΛΑΓΟΥ (*LEPUS EUROPAEUS*) ΩΣ ΠΗΓΗ ΑΝΑΔΥΟΜΕΝΩΝ ΚΑΙ ΕΠΑΝΑΔΥΟΜΕΝΩΝ ΠΑΘΟΓΟΝΩΝ ΠΑΡΑΓΟΝΤΩΝ

Διδακτορική Διατριβή της

Κωνσταντίνας Ν. Τσοκανά

## Περίληψη

Η παρούσα διατριβή, είχε στόχο τη διερεύνηση του ρόλου του Καφέ Ευρωπαϊκού λαγού (*Lepus europaeus*), ως πηγή σημαντικών αναδυόμενων και επαναδυόμενων παθογόνων παραγόντων. Οι ορολογικές και μοριακές εξετάσεις που διεξήχθησαν, αποτέλεσαν την πρώτη διερεύνηση της παρουσίας μόλυνσης ή / και της έκθεσης του Καφέ Ευρωπαϊκού λαγού σε παρασιτικούς και βακτηριακούς παθογόνους παράγοντες, συμπεριλαμβανομένων των *Toxoplasma gondii*, *Neospora caninum*, *Leishmania infantum*, *Bartonella* spp, *Brucella* spp, *Coxiella burnetii* και *Francisella tularensis* στην Ελλάδα. Επιπλέον, καθορίστηκε η οικολογική θέση των οροθετικών λαγών στα πρωτόζωα *T. gondii* και *L. infantum*. Ο ρόλος αυτού του είδους στην επιδημιολογία της λεϊσμανίασης, διερευνήθηκε περαιτέρω με τη χρήση Συστημάτων Γεωγραφικών Πληροφοριών (ΣΓΠ) για την αναγνώριση των περιβαλλοντικών παραμέτρων, οι οποίες σχετίζονται με τη μόλυνση των λαγών από τα είδη του πρωτόζωου *Leishmania* καθώς και μέσω της φυλογενετικής ανάλυσης των αλληλουχιών των πρωτόζωων *Leishmania*

που ανιχνεύθηκαν σε λαγούς. Η φυλογενετική θέση των πρωτόζωων *Leishmania* spp που ανιχνεύθηκαν σε ανθρώπους, σκύλους, γάτες και λαγούς στην περιοχή της Θεσσαλίας, καθώς και η πρώτη ένδειξη για την κυκλοφορία της *L.donovani* στην Ελλάδα αποτελούν επίσης μέρος αυτής της διατριβής.

Στο πρώτο κεφάλαιο, έγινε μια ανασκόπηση των εργαλείων που έχουν αναπτυχθεί για τη μοριακή διάγνωση της Λεϊσμανίασης, την ταυτοποίηση των ειδών και τη φυλογενετική τους ανάλυση, εργαλεία τα οποία παρουσιάζουν διαφορετική ευαισθησία και διακριτική ισχύ.

Η πρώτη αναφορά της παρουσίας μόλυνσης από είδη του πρωτόζωου *Leishmania* στην Ελλάδα, γίνεται στο δεύτερο κεφάλαιο αυτής της διατριβής. Ο επιπολασμός μόλυνσης από πρωτόζωα *Leishmania* στους λαγούς βρέθηκε να είναι 23.49% στη Βόρεια Ελλάδα (νομοί Θεσσαλονίκης και Χαλκιδικής) από το 2007-2011. Η φυλογενετική ανάλυση, έδειξε ότι οι αλληλουχίες των πρωτόζωων *Leishmania* που ανιχνεύθηκαν σε λαγούς, ανήκουν στο σύμπλεγμα *Leishmania donovani.* Η ομολογία των αλληλουχιών των νουκλεοτιδίων μεταξύ των πρωτόζωων *Leishmania* που ανιχνεύθηκαν σε σκύλους της ίδιας περιοχής και αυτών που ανιχνεύθηκαν σε λαγούς ήταν 98,9%, υποδηλώνοντας την πιθανή αλληλεπικάλυψη του άγριου και οικόσιτου κύκλου μετάδοσης του πρωτόζωου *Leishmania* spp στην περιοχή. Δεν υπήρξαν ενδείξεις σύνδεσης των περιστατικών Λεϊσμανίασης σε ανθρώπους και των μολυσμένων λαγών στους νομούς που μελετήθηκαν. Η υπόθεση που διατυπώθηκε ήταν ότι στην περιοχή της Χαλκιδικής επικρατεί ο άγριος κύκλος μετάδοσης της Λεϊσμανίασης, ενώ στην περιοχή της Θεσσαλονίκης φαίνεται να υπάρχει αλληλεπικάλυψη του άγριου και οικόσιτου κύκλου μετάδοσης του πρωτόζωου *Leishmania*. Η βροχόπτωση αναγνωρίστηκε ως η περιβαλλοντική παράμετρος που επηρεάζει την παρουσία του DNA του πρωτόζωου *Leishmania* στους λαγούς, καθώς η αύξηση της βροχόπτωσης κατά μια μονάδα, αναμένεται να αυξήσει την πιθανότητα παρουσίας λαγών μολυσμένων με το πρωτόζωο *Leishmania* κατά 6,1%.

Το τρίτο κεφάλαιο, αφορά στην μοριακή και ορολογική διερεύνηση για την παρουσία μόλυνσης ή / και έκθεσης των Καφέ Ευρωπαϊκών λαγών από τη Βόρεια και Κεντρική Ελλάδα, σε παρασιτικούς παθογόνους παράγοντες κατά τη διάρκεια θηρευτικών περιόδων δύο ετών, η οποία έδειξε ότι το 5.7%, 0.95% και 12.4% των λαγών αντέδρασαν θετικά στην παρουσία αντισωμάτων έναντι των *T.gondii*, *N.caninum* και

*L.infantum*. Αντίστοιχα, οι εξεταζόμενοι λαγοί ήταν μολυσμένοι με τα πρωτόζωα *N.caninum* (3,8%) και *L.infantum* (9,6%). Η ανάλυση με ΣΓΠ σε συνδυασμό με το Οικολογικό Μοντέλο Θέσης έδειξαν ότι η βροχόπτωση και οι χρήσεις γης επηρεάζουν σημαντικά την έκθεση του λαγού στα πρωτόζωα *T.gondii* και *L.infantum*. Επιπλέον, εντοπίστηκαν οι περιοχές υψηλού κινδύνου για την έκθεση του λαγού σε *T.gondii* και *L.infantum* και δημιουργήθηκε ένας χάρτης πρόβλεψης του κινδύνου έκθεσής τους στα εν λόγω πρωτόζωα για τις περιοχές της βόρειας και κεντρικής Ελλάδας.

Η φυλογενετική θέση των πρωτόζωων *Leishmania* spp που ανιχνεύθηκαν σε ανθρώπους, σκύλους, γάτες και λαγούς στη Θεσσαλία, προσδιορίστηκε μέσω της φυλογενετικής ανάλυσης και της ανάλυσης της περιοχής ITS1 του γενώματος του πρωτόζωου *Leishmania* και περιγράφεται στο τέταρτο κεφάλαιο αυτής της διατριβής. Οι αλληλουχίες *Leishmania* που προήλθαν από διαφορετικούς ξενιστές βρέθηκε ότι ανήκουν στο σύμπλεγμα *Leishmania donovani*. Το είδος *L. infantum* εντοπίστηκε σε όλες τις αλληλουχίες *Leishmania* που συμπεριλήφθηκαν στη μελέτη, υποδεικνύοντας ότι αποτελεί το κυρίαρχο είδος που κυκλοφορεί σε διαφορετικούς ξενιστές στη Θεσσαλία. Επίσης, την ύπαρξη του ζωονοτικού κύκλου μετάδοσης της *L.infantum* στην περιοχή μελέτης και την πιθανή αλληλεπικάλυψη μεταξύ του οικόσιτου και του άγριου κύκλου μετάδοσης του πρωτόζωου. Απροσδόκητα, μία αλληλουχία *Leishmania* ταυτοποιήθηκε ως *L. donovani* σε ένα δείγμα γάτας, υποδεικνύοντας την πιθανή παρουσία μόλυνσης από *L. donovani*, σε μια γάτα στην περιοχή της μελέτης, δίνοντας το έναυσμα για περαιτέρω διερεύνηση.

Το τελευταίο κεφάλαιο ,αφορά στην εμφάνιση μόλυνσης ή / και έκθεσης του Καφέ Ευρωπαϊκού λαγού, σε βακτηριακούς παθογόνους παράγοντες συμπεριλαμβανομένων των *Bartonella* spp, *Brucella* spp, *Coxiella burnetii* και *Francisella tularensis*. Η διερεύνηση που πραγματοποιήθηκε κατά τη διάρκεια θηρευτικών περιόδων δύο ετών, στη βόρεια και κεντρική Ελλάδα. Αντισώματα κατά της *Bartonella* spp ανιχνεύθηκαν στο 11,4% των εξεταζόμενων λαγών, ενώ κανένα δείγμα δεν βρέθηκε οροθετικό για τα βακτήρια *Brucella* spp, *C. burnetii* και *F. tularensis*. Η παρουσία του DNA των βακτηρίων *Bartonella* spp, *Brucella* spp, *C. burnetii* και *F. tularensis* δεν ανιχνεύθηκε στα δείγματα που εξετάστηκαν. Φαίνεται ότι ο ρόλος του Καφέ Ευρωπαϊκού λαγού ,στην επιδημιολογία των παθογόνων μικροοργανισμών *Brucella* spp, *C. burnetii* και *F. tularensis* είναι ελάχιστης σημασίας στην Ελλάδα ενώ το

γεγονός ότι το είδος αυτό, βρέθηκε να έχει εκτεθεί στη *Bartonella* spp αξίζει περαιτέρω διερεύνηση για την αναγνώριση του ρόλου του ως εναλλακτικού ξενιστή αυτού του βακτηρίου.

Η παρούσα διατριβή, παρέχει μια εικόνα για το ρόλο ενός σημαντικού είδους θηραμάτων στην Ελλάδα, του Καφέ ευρωπαϊκού λαγού, στην επιδημιολογία παρασιτικών και βακτηριακών ζωονοτικών παθογόνων παραγόντων. Το είδος αυτό έχει περιορισμένη περιοχή ενδημίας, είναι επομένως επιρρεπές σε παθογόνους παράγοντες που υπάρχουν στο φυσικό του περιβάλλον. Ωστόσο, μοιράζεται τον ίδιο χώρο διαβίωσης με άλλα άγρια και ελεύθερης εκτροφής ζώα, συμπεριλαμβανομένων των κυνοειδών, των αιλουροειδών και των μεγάλων αρπακτικών πτηνών, που αποτελούν τους φυσικούς του θηρευτές καθώς επίσης και με τους κυνηγετικούς σκύλους. Η σπουδαιότητά του, ενισχύεται περαιτέρω, λόγω των μετακινήσεων ζώντων ζώων τόσο σε διασυνοριακό επίπεδο όσο και σε μεγάλες αποστάσεις, καθώς επίσης και λόγω του ότι αποτελεί ξενιστή διαβιβαστών και θήραμα πολλών σαρκοφάγων και παμφάγων, τα οποία μετακινούνται καλύπτοντας εκτεταμένες αποστάσεις και έρχονται σε επαφή με τα κατοικίδια ζώα και τον άνθρωπο. Εξάλλου, η σύντομη διάρκεια ζωής του, καθιστά αυτό το είδος, έναν εξαιρετικό δείκτη για την πρόσφατη μετάδοση παθογόνων παραγόντων στην περιοχή. Η εντατική επαφή του, με διαβιβαστές όπως κρότωνες, κουνούπια και σκνίπες, έχει εμπλέξει τους λαγούς στην επιδημιολογία σημαντικών νοσημάτων που προκαλούνται από διαβιβαστές όπως η Λεϊσμανίαση. Η συνεχής επιτήρηση των πληθυσμών των λαγών, θα μπορούσε να παρέχει πληροφορίες σχετικά με την κατάσταση της υγείας του πληθυσμού του λαγού, καθώς και τους παθογόνους παράγοντες που κυκλοφορούν στην περιοχή διαβίωσής του. Παθογόνοι παράγοντες, οι οποίοι συνιστούν κίνδυνο για την άγρια πανίδα, τα κατοικίδια ζώα και φυσικά τον άνθρωπο.